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Fermentations

INTRODUCTION

This chapter presents the primary chemical principles, laws, and generalizations which govern fermentations in milk and milk products. In the selection of topics, richness and profundity in chemical content have been primary considerations. Although stress is placed on intermediary metabolism, the utilitarian aspects of lacteal fermentations have not been ignored. A balance is sought between the theoretical and the practical.

Fermentation is a term with many shades of meaning, each of which is definitive with respect to limited biochemical changes brought about by microorganisms or their enzyme systems. Prescott and Dunn[407] have reviewed the changes of meaning which the term has undergone since its derivation as a descriptive word for a gentle and "boiling" condition observed in wine-making. They define fermentation in a broad sense as "a process in which chemical changes are brought about in an organic substrate, whether carbohydrate or protein or fat or some other type of organic material, through the action of biochemical catalysts known as 'enzymes' elaborated by specific types of living microorganisms." The fermentation phenomena encountered in the field of dairy technology fall within the scope of this definition, and may be subdivided into desirable and undesirable fermentations. The desirable fermentations leading to the formation of useful intermediate or end products are usually brought about by the inoculation of milk or its by-products with pure or with mixed cultures; the undesirable ones leading to the formation of substances deleterious to milk or its products are caused at times by organisms constituting the natural flora of milk, or by contaminants.

A more restrictive definition has been proposed. According to Elsden,[107] "Fermentation may be defined as a biological process in which chemical energy is made available for growth by oxidative reactions, the ultimate hydrogen acceptors for which are substances

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other than O_2." This definition rather than the broader one is more suitable to the discussion of the intermediate products of the metabolism of carbohydrates in yeast and muscle. The reactions leading to the formation of the intermediates are well established and furnish the theoretical basis for the general study of component reactions in bacterial fermentations. Elsden has enunciated the thesis that the primary mechanism found in muscle and yeast, the operation of which results in the formation of pyruvic acid, is found also in those bacteria which ferment carbohydrates, and that such differences in the end products as are observed result from the ability of bacteria to synthesize a wide range of hydrogen acceptors from pyruvic acid.

THEORIES AND METABOLIC PROCESSES OF FERMENTATION

Under conditions in which one organism with simple nutritional requirements predominates over all others and overgrows them, the fermentation in milk will parallel that in pure culture. The usual effect, however, is one in which varying results are obtained because of variations in bacterial strains, in the milk supply, and in its treatment, and finally in external conditions such as temperature, oxygen supply, acidity, etc.

Details of fermentations are important and empirical formulations are indispensable in the art and practice. These will be discussed in the second and third sections. The present section, however, is devoted to the generalizations brought to light as a result of studies on isolated systems containing the components of cell-free extracts and their specific substrates. The reactions, reaction products, and reaction mechanisms observed in such systems occur in lacteal fermentations, but possessing a general character, their occurrence is not confined to any particular fermentation medium or substrate.

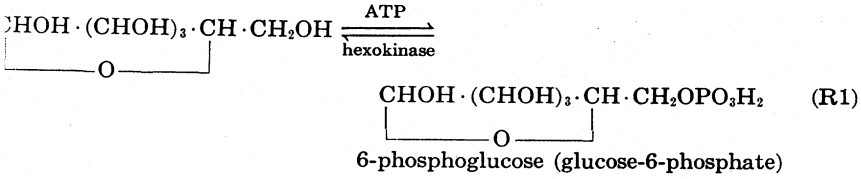
Topics will be discussed in the following order: the Meyerhof-Embden pathway to pyruvic acid; the pentose phosphate pathway to pyruvic acid; the lactic acid fermentation; the alcohol fermentation, the glycerol fermentation; the propionic acid fermentation; the oxidative decarboxylation of pyruvic acid; the *Escherichia coli* fermentation; the *Aerobacter* type fermentation; the butyric and related fermentations; fat metabolism; the citric acid cycle; "energy-rich" bonds and their formation; polysaccharide synthesis and assimilation. The order listed is a logical one for each topic flows out of the preceding one.

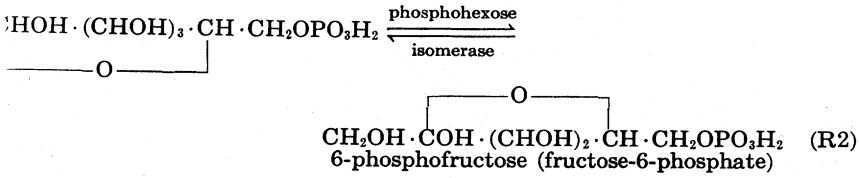
The Meyerhof-Embden Pathway

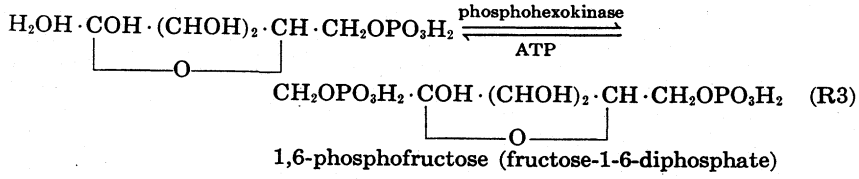
The Meyerhof-Embden pathway constitutes a generalization of great importance, as significant in bacterial processes as it is in the

yeast fermentation and in glycolysis. The pathway may conveniently be divided into six parts, the first leading to the formation of fructose-1-6-diphosphate, the second to the phosphotrioses, phosphodihydroxyacetone, and 3-phosphoglyceraldehyde, the third to 3-phosphoglycerate, the fourth to 2-phosphoglycerate, the fifth to phosphoenolpyruvate, and the sixth to pyruvic acid. The reactions are listed herewith:

Formation of Fructose-1,6-Diphosphate.—The first stage comprises the following series of three reactions:[44,75,176,177,366,434,545,551]



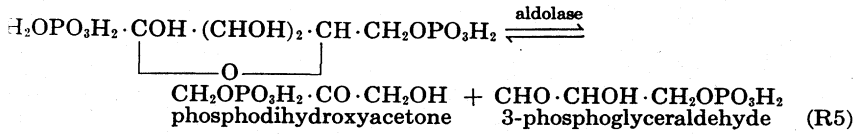




A more efficient mechanism for the conversion of fructose-6-phosphate to fructose-1-6-diphosphate has been proposed.[310]

2 fructose-6-phosphate $\rightleftharpoons$ fructose diphosphate + glucose (R4)

The Second Stage: Phosphotriose Formation[17,342–344,511]



$$CHO \cdot CHOH \cdot CH_2OPO_3H_2 \overset{\text{isomerase}}{\rightleftharpoons} \underset{\text{phosphodihydroxyacetone}}{CH_2OH \cdot CO \cdot CH_2OPO_3H_2} \quad \text{(R6)}$$

The Third Stage: 3-Phosphoglycerate Formation.—The third stage consists of two steps, the first leading to the formation of 1,3-diphosphoglycerate and the second to 3-phosphoglycerate, thus:[53,345,363,515]

$$CH_2OPO_3H_2 \cdot CHOH \cdot CHO + DPN^+ + H_3PO_4 \overset{\text{phosphoglyceraldehyde dehydrogenase}}{\rightleftharpoons} \underset{\text{1,3-diphosphoglyceric acid}}{CH_2OPO_3H_2 \cdot CHOH \cdot CO_2 \sim PO_3H_2} + DPNH + H^+ \quad \text{(R7)}$$

$$COO \sim PO_3^{2-} \cdot CHOH \cdot CH_2OPO_3^{2-} + ADP^{2-} \overset{\text{3-phosphoglycerate kinase}}{\rightleftharpoons} \underset{\text{3-phosphoglycerate}}{COO^- \cdot CHOH \cdot CH_2OPO_3^{2-}} + ATP^{3-} \quad \text{(R8)}$$

The Fourth Stage: 2-Phosphoglycerate Formation.[476]

$$COOH \cdot CHOH \cdot CH_2OPO_3H_2 \overset{\text{phosphoglycero-mutase}}{\rightleftharpoons} \underset{\text{2-phosphoglyceric acid}}{COOH \cdot CHOPO_3H_2 \cdot CH_2OH} \quad \text{(R9)}$$

$$COOH \cdot CHOH \cdot CH_2OPO_3H_2 + \underset{\text{2,3-phosphoglyceric acid}}{COOH \cdot CHOPO_3H_2 \cdot CH_2OPO_3H_2} \overset{\text{phosphoglycero-apomutase}}{\rightleftharpoons} COOH \cdot CHOPO_3H_2 \cdot CH_2OH + COOH \cdot CHOPO_3H_2 \cdot CH_2OPO_3H_2 \quad \text{(R10)}$$

The Fifth Stage: Phosphoenolpyruvate Formation.[316,516]

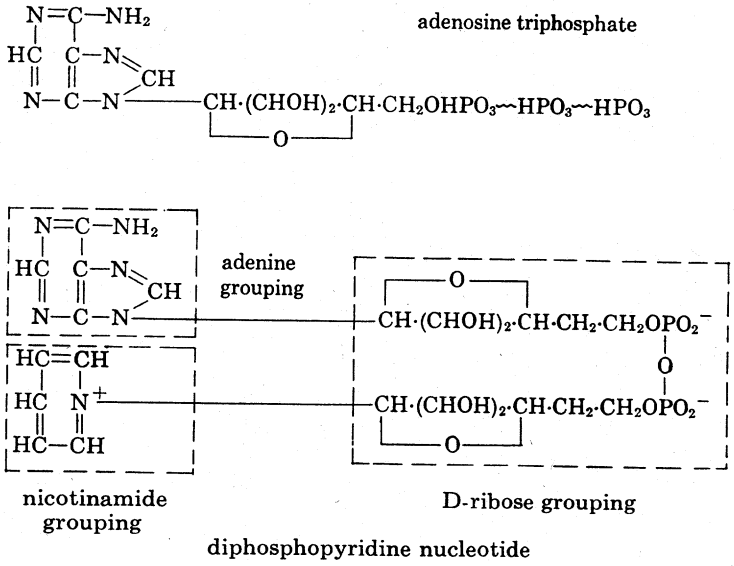
$$COOH \cdot CHOPO_3H_2 \cdot CH_2OH \overset{\text{enolase, } -H_2O}{\underset{+H_2O}{\rightleftharpoons}} \underset{\text{phosphoenolpyruvic acid}}{COOH \cdot CO \sim PO_3H_2 : CH_2} \quad \text{(R11)}$$

The Sixth Stage: Pyruvate Formation.[32,43,274,279,346]

$$COOH \cdot CO \sim PO_3H_2 : CH_2 + ADP \overset{\text{pyruvate kinase}}{\rightleftharpoons} \underset{\text{Pyruvic acid}}{COOH \cdot CO \cdot CH_3} + ATP \quad \text{(R12)}$$

Pyruvate is a key intermediate whose importance will become clear in the ensuing discussion. In the steps leading to its formation, a number of enzymes and coenzymes have been involved. Many of the enzymes have been isolated in crystalline form. Two important coenzymes make their appearance for the first time. One is adenosine triphosphosphate (ATP), the other diphosphopyridine nucleo-

tide (DPN), currently known as nicotinamide-adenine-dinucleotide (NAD). The structural formulas are given below:



diphosphopyridine nucleotide

The Pentose Phosphate Pathway

It is generally accepted that the Meyerhof-Embden pathway is not unique for bacteria, and that among others there are two branches (the oxidative and the nonoxidative) of a metabolic route which conjoins with the Meyerhof-Embden scheme at a number of points, and which is known as the pentose phosphate pathway. The oxidative pathway is followed by many heterolactic bacteria among which are several species active in the production of biacetyl, the aroma-producing substance in starter cultures. The pathway comprises the following steps: The oxidation of 6-phosphoglucose to the α-lactone of 6-phosphogluconic acid in the presence of Warburg's (Zwischenferment) 6-phosphoglucose dehydrogenase,[77,379,513] and the coenzyme triphosphopyridine nucleotide (TPN),* also known as coenzyme II; the hydrolysis of the lactone to 6-phosphogluconic acid in the presence of a lactonase; the oxidative decarboxylation of 6-phosphogluconic acid to ribulose-5-phosphate in the presence of 6-phosphogluconic dehydrogenase (decarboxylating) and TPN; the isomerization of ribulose-5-phosphate to ribose-5-phosphate in the presence of

*TPN is related structurally to DPN above containing an additional phosphate group bound presumably to the adenosine part of the molecule.

phosphoriboisomerase (this step is only necessary when ribose-5-phosphate is the substrate);[67–69,217,308,457] the epimerization of ribulose-5-phosphate to xylulose-5-phosphate in the presence of phosphoketopentoepimerase; the phosphorolytic cleavage of xylulose-5-phosphate to acetyl phosphate and 3-phosphoglyceraldehyde in the presence of phosphoketolase.[191,221] With the formation of phosphoglyceraldehyde, the pentose and the Meyerhof-Embden pathways merge enroute to the formation of pyruvic acid. The active two carbon compound, acetyl phosphate, is an extremely important intermediate about which much more will be recorded elsewhere in this chapter.

The Nonoxidative Pentose Phosphate Pathway

This pathway is distinguished by its requirement for "active glycolaldehyde" donors and acceptors. In pentose synthesis, glyceraldehyde-3-phosphate is the acceptor and sedoheptulose-7-phosphate and fructose-6-phosphate are important donors, thus:[215]

$$\underset{\text{ribulose phosphate}}{CH_2OH\cdot CO\cdot (CHOH)_2\cdot CH_2OPO_3H_2} \xrightleftharpoons{\text{transketolase}} H_2COH\cdot CO\cdot (CHOH)_2\cdot CH_2OPO_3H_2 + \underset{\text{phosphoglyceraldehyde}}{CHO\cdot CHOH\cdot CH_2OPO_3H_2} \quad \text{(R13)}$$

$$\underset{\text{ribulose-5-phosphate}}{CH_2OH\cdot CO\cdot (CHOH)_2\cdot CH_2OPO_3H_2} \xrightleftharpoons{\text{isomerase}} \underset{\text{ribose-5-phosphate}}{CHO\cdot (CHOH)_2\cdot CH_2OPO_3H_2} \quad \text{(R14)}$$

$$\underset{\text{ribose-5-phosphate}}{CHO\cdot (CHOH)_3\cdot CH_2OPO_3H_2} + H_2COH\cdot CO\cdot (CHOH)_2\cdot CH_2OPO_3H_2 \rightleftharpoons \underset{\text{sedoheptulose-6-phosphate}}{CH_2OH\cdot CO\cdot (CHOH)_4\cdot CH_2OPO_3H_2}$$

(enzyme)

and in a similar manner, fructose-6-phosphate serves as a donor to the acceptor glyceraldehyde-3-phosphate yielding ribulose-5-phosphate and erythrose-4-phosphate. Not only is sedoheptulose formed in a transketolase catalyzed reaction, but it is also formed in a transaldolase mediated reaction between fructose-6-phosphate and erythrose-4-phosphate.

The preparation and properties of transketolase have been described in a number of publications.[85,218,416,417,469] Transaldolase has been obtained from yeast in a highly purified state.[215]

The Lactic Acid Fermentation

Knowledge of the several metabolic pathways is a prerequisite to a clear understanding of bacterial fermentations. This observation applies quite forcefully to the lactic acid fermentation. Lactic acid, the most common of all substances produced microbially in milk, develops naturally (although uncontrollably) in raw milk since lactic acid bacteria are usually present. Lactic acid is produced in the manufacture of virtually all cultured products, for example, in the production of buttermilk, yoghurt, and kefir among cultured milks, and Cottage, Cheddar, Swiss, and Brick, among the cheeses.

Description of the Lactic Acid Bacteria.—The lactic acid bacteria are gram positive, nonspore-forming, nonmotile, and almost always catalase-free. The homofermentative types produce lactic acid from sugar in yields ranging from 80 to 98%, and small quantities of other products. Included among these types are species of the genus *Streptococcus*, and the subgenera, *Streptobacterium*, and *Thermobacterium*. *Streptococcus* species produce dextro-lactic acid in concentrations up to 1%; *Streptobacterium* species produce, at an optimum temperature of 30°C., either dextro- or inactive lactic acid in concentrations up to 1.5%; *Thermobacterium* species produce, at optimum temperatures of 40°C. or higher, levo- or inactive lactic acid in concentrations up to 3%. *Lactobacillus bulgaricus*† in yoghurt, *Streptococcus lactis* in cultured buttermilk and cheese are important homofermentative types encountered in dairy technology.

The heterofermentative lactic acid bacteria ferment glucose to form CO_2, alcohol, and acetic acid, in addition to lactic acid. Comprising this group are species of *Lactobacillus*, which produce inactive lactic acid, and species of *Leuconostoc*, which usually develop alcohol, CO_2, and limited amounts of lactic and acetic acids. Levo-lactic acid is always produced, and simultaneously dextro-lactic acid is sometimes formed. About 25% of glucose may be converted to CO_2. The flavor-producing organisms of butter and cultured buttermilk, *Leuconostoc citrovorum* and *Leuconostoc dextranicum*, belong to this group. Based on the observation that alcohol, carbonic-, and acetic acids are produced in significant quantities by *Lactobacillus bifidus* and members of the genus *Pediococcus*, it would appear that these bacteria should be classified with the heterolactics. However, recent studies with radioactive glucose indicate that in common with homofermentative types, the aforementioned bacteria ferment glu-

† In bacteriological nomenclature, the names of the genera are spelled out wherever they occur for the first time. Thereafter they are identified by their first letter.

cose according to the Meyerhof-Embden scheme.[50,235] Hence *L. bifidus* and members of the genus *Pediococcus* have been classified with the homolactics.

The distinction between the homofermentative and heterofermentative lactic acid bacteria is not a hard and fast one. In the experiments of Friedemann,[124] a strain of the homofermentative organism *Streptococcus faecalis* produced 74, 14 ,6, and 7 mM., respectively, of lactate, formate, acetate, and ethanol per 50 mM. of glucose, whereas the heterofermentative lactic acid organism *Leuconostoc mesenteroides* produced 81 mM. lactate, 4 mM. acetate, and 10 mM. ethanol. Clearly the distinction here is not so much in the ratio between lactate and other products as it is in the ratio between formate and ethanol. Alkaline media favor the formation of products other than lactic acid. *Streptococcus faecalis* var. *liquefaciens* produces 87% acid at pH 5, and only 61% at pH 9.[155]

The homolactics are actually quite responsive to altered conditions of fermentation and to altered substrates.[153] The use of oxidized substrate diverts the lactic fermentation to dismutation and to acetylmethylcarbinol (acetoin) formation; the use of reduced substrate diverts it to a coupled lactate-succinate fermentation. An alkaline medium in glucose dissimilation favors the formation of formate, acetate, and ethanol.

Recently Platt and Foster[396] observed that *Streptococcus cremoris* produced anaerobically in a glucose medium subject to no pH control, acetic and formic acids, CO_2 and ethanol in addition to lactic acid, and in a medium held at pH 7.0 an increased number of products. *S. lactis* produced some acetoin but no formic acid in the absence of pH control, and products altered in character at pH 7.0. Thus in a medium sparged with nitrogen, lactic, acetic and formic acids, CO_2, ethanol, biacetyl, acetoin and 2,3-butanediol were found. *Streptococcus thermophilus* produced the combined products developed by *S. cremoris* and *S. lactis* in the absence of pH control, and products altered in kind at pH 7.0. Harvey[182] noted that *S. lactis* and *S. cremoris* were also able to develop acetaldehyde and acetone.

A surprising result was obtained by Steele *et al.*,[471] who, while studying capsular polysaccharide synthesis by Group A streptococci, made the observation that a typically heterolactic instead of a homolactic fermentation was obtained when galactose was used as a carbon source.

Species of the genus *Lactobacillus* may be homofermentative, belonging then to the subgenus *Thermobacterium* or the subgenus *Streptobacterium*, or they may be heterofermentative, belonging then to

the subgenus *Betabacterium*. An interesting organism is *Lactobacillus pentosus*. It converts pentoses into lactic and acetic acids in equimolar proportions. Other pentose-decomposing organisms break down the sugar with the production of relatively greater quantities of lactic acid, thus:

$$6C_5H_{10}O_5 \rightarrow \underset{\text{lactic acid}}{8C_3H_6O_3} + \underset{\text{acetic acid}}{3C_2H_4O_2} \qquad \text{(R15)}$$

The *L. pentosus* fermentation has been studied with 1-C^{14} labeled L-arabinose and xylose. Cleavage occurred between 2-C and 3-C. Labeled carbon was found in the methyl group of acetic acid, indicating that a 2-keto-pentose was the precursor. The study of this fermentation has contributed much to our understanding of intermediary metabolism.

Intermediates in the Homofermentative Lactic Fermentation.—The metabolic route followed in the production of lactic acid by homolactic bacteria is believed to parallel that followed in glycolysis, i.e., in the production of lactic acid from glucose in muscle. The conclusiveness with which parallel pathways to pyruvic acid in muscle and yeast metabolism have been demonstrated supports the hypothesis that the production of lactic acid in a homolactic fermentation proceeds according to the Meyerhof-Embden mechanism. This would mean that the lactic acid fermentation follows the pathway of the alcohol fermentation up to the point at which pyruvic acid is produced. The lactic acid fermentation diverges at this point. Lacking pyruvic apocarboxylase but possessing lactic apodehydrogenase,[341] the lactic acid bacteria utilize reduced coenzyme I for the reduction of pyruvic to lactic acid.

Gibbs *et al.*[136] carried out investigations with *Lactobacillus casei* and 1-C^{14} labeled glucose. The lactate resulting from the fermentation contained all of its radioactivity in the methyl group, a result predicted by the Meyerhof-Embden scheme. Kuhn and Tiedemann[275] investigated the metabolism of *L. bifidus* and the conversion of 1-C^{14} labeled glucose into radioactive products. It is known that in the *L. bifidus* fermentation of glucose, acetic and lactic acids are formed in approximately equal quantities, and CO_2 is not found. Kuhn and Tiedemann found radioactivity equally distributed between acetic and lactic acids. However, in conformity with the Meyerhof-Embden scheme, activity was associated with the methyl, but not with the carboxyl group. Incorporation of $CH_3 \cdot C^{14}OOH$ into the medium resulted in the formation of lactic acid with labeled α-carbon. The acetic and lactic acids appeared to possess a common precursor. Cell preparations showed aldolase and lactic dehydro-

genase activity. If, in glucose fermentation, homofermentative lactic acid bacteria follow the Meyerhof-Embden route, the required enzymes should be present in these bacteria. Aldolase, a key enzyme, has been demonstrated to be present in extracts prepared from *S. faecalis*, *S. lactis*, a so-called strain of *L. citrovorum* (actually a *Pediococcus* species), *Lactobacillus parabifidus*, *L. bulgaricus*, *Lactobacillus delbrückii*, *Lactobacillus plantarum*, *Lactobacillus arabinosus*, *Lactobacillus leichmannii*, *Lactobacillus bifidus*, and *Microbacterium lacticum*.[51,275,497] Hexokinase appears in extracts prepared from *L. bulgaricus* and *M. lacticum*;[444,497] and phosphohexoseisomerase, phosphohexokinase, phosphotrioseisomerase, phosphoglyceraldehyde dehydrogenase, phosphoglyceromutase, pyruvatekinase, and enolase appear in extracts prepared from *M. lacticum*.[497] Lactate dehydrogenase and phosphoglyceraldehyde dehydrogenase have been found in extracts prepared from the heterofermentative organism *L. mesenteroides*, yet the key enzymes belonging to the Meyerhof-Embden scheme, aldolase and phosphotrioseisomerase, have not been found.[51,94,95]

Intermediates in the Heterolactic Fermentation.—Von Baeyer observed that oxygen tends to move in organic compounds toward the carbon richest in oxygen. In glucose, the carbon of the aldehyde group, 1-C, is richest in oxygen. The rule of von Baeyer is not followed in the Meyerhof-Embden scheme. The CO_2 produced in the alcohol fermentation, and the carboxyl group produced in the lactic fermentation, derive not from the oxygen-rich 1-C, but rather from 3-C and 4-C. This situation, of course, prevails because isomerization reactions occur, mediated by phosphoglucoisomerase and phosphotriose isomerase. It has already been pointed out that the homolactic fermentation probably follows the Meyerhof-Embden pathway. However, with respect to the heterolactics generally, no unique pathway exists. Metabolism of glucose by *L. bifidus* appears to follow the Meyerhof-Embden scheme; however, metabolism of glucose by *L. mesenteroides* clearly follows an alternate pathway, that of the oxidative pentose phosphate route, and moreover, one conforming to the von Baeyer rule. With a strain of *L. mesenteroides*, 1-C^{14} labeled glucose gave rise to labeled CO_2, and 3,4-C^{14} labeled glucose gave rise to carbinol-C labeled ethanol and carboxyl-C labeled lactate in equal proportions.[94,154] The methyl carbon of ethanol and the α and β-carbons of lactate were unlabeled, and were presumed to arise from the 2,5,6,-carbons of glucose. The overall reaction is expressed thus:

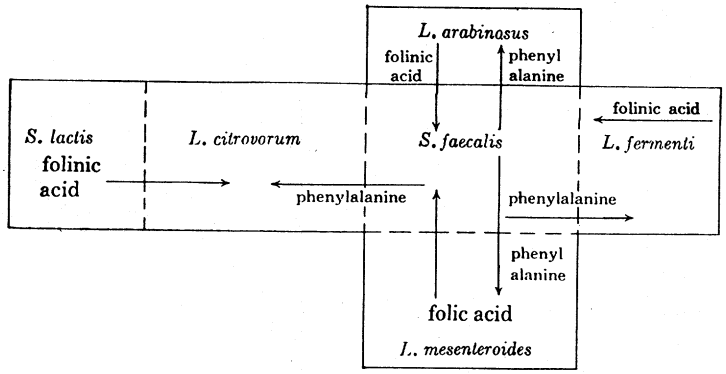
$$C_6H_{12}O_6 \rightarrow CO_2 + C_2H_5OH + CH_3 \cdot CHOH \cdot CO_2H \qquad \text{(R16)}$$

One mole of glucose always yielded one mole of CO_2, one mole of ethanol, and one mole of lactate. The presence of aldolase and isomerases in cell extracts was not demonstrable. On the other hand, enzymes linking the reactions leading from 3-phosphoglyceraldehyde to D(−) lactic acid, as in the Meyerhof-Embden scheme, were found. Furthermore, 6-phosphoglucose dehydrogenase was found in *L. mesenteroides*.[95]

These findings may be explained in terms of the reaction pattern of the pentose phosphate scheme. Actually, an impetus for the exploration of this alternate route to pyruvic acid was provided by the work on the *Leuconostoc* fermentation. On examination of the reactions comprising this pathway, it may readily be seen that the carbon in CO_2 would derive from the 1-C of glucose; the carboxyl carbon of lactic acid and the carbinol carbon of ethanol would derive from the 3,4-C of glucose; and finally the methyl carbon of lactic acid and of ethanol would derive from the 5,6-C of glucose.

The Optical Configuration of Lactic Acid.—Certain lactobacilli, which normally produce DL(±) lactic acid, will when grown in a niacin deficient medium synthesize the D(−) acid.[244] Inasmuch as DPN, a niacin derivative, is a cofactor of racemase, it appears that racemase activity would be seriously reduced in media deficient in niacin; and hence the DL(±) acid would not accumulate in normal proportions. The occurrence of specific lactic dehydrogenases for the synthesis of D(−) and DL(±) lactic acid would account for the observation that the configuration of lactic acid often serves to distinguish between species.

Associated Growth.—Lactic acid bacteria are extremely fastidious in their growth requirements. It often happens that species grown in association will thrive under conditions in which one or more members if cultured individually will fail to grow. Exceedingly interesting experiments to illustrate the relationship between symbiotic growth, and the requirement for specific metabolites have been described by Nurmikko,[382] and independently by Koft and Morrison.[253] Using a medium which was complete only for a strain of *S. lactis*, deficient, however, in phenylalanine for *L. citrovorum*, *L. arabinosus*, *Lactobacillus fermenti* and *L. mesenteroides*, and deficient moreover, in folic acid for *L. citrovorum* and *S. faecalis*, Nurmikko compartmentalized individual species in one or another of six cells while at the same time he provided for exchange of dialyzable components according to the scheme shown on p. 684. Under these conditions all species thrived; for the missing growth factors for any one organism were supplied by one or more of the remaining species.



The Propionic Acid Fermentation

The propionic acid bacteria are gram positive, nonspore-forming, nonmotile rods. Like the homolactics, they grow under anaerobic and microaerophilic conditions. They ferment lactic acid, carbohydrates, and polyalcohols with the formation of propionic and acetic acids and carbon dioxide. They are usually strongly catalase positive, and contain a cytochrome system. Their vitamin requirements have been reported.[487]

Pathway to Propionic Acid.—In the manufacture of certain types of cheese, such as Swiss, starter microorganisms convert the lactose of milk into lactic acid, and it is only after this conversion is practically complete that the propionic acid bacteria enter the fermentation and dissimilate lactic acid as follows:

$$3\ CH_3\cdot CHOH\cdot CO_2H \rightarrow 2\ \underset{\text{propionic acid}}{CH_3\cdot CH_2\cdot CO_2H} + \underset{\text{acetic acid}}{CH_3\cdot CO_2H} + CO_2 \qquad \text{(R17)}$$

This general formulation is given by Fitz.[120] Van Niel[500] found the ratio between acetic acid and CO_2 conform, to the theoretical value of one, but not the ratio between propionic and acetic acids, which he found to lie between 1.6 and 1.8. It is very likely that the mechanism involved in the formation of lactic acid from carbohydrate is the same in the propionic and in the homolactic fermentations. In this connection the components of propionic bacteria may be considered. Hexose phosphates, for example, are present in dried cells.[503] The phosphorylation mechanism via ATP is present; and glucose, glycerol, arabinose, and erythritol may undergo phosphorylation. Phosphoglyceric acid has been demonstrated to form in systems containing the following: toluene-treated cells, hexose diphosphate or hexose, and a hydrogen acceptor such as pyruvate.[527]

The Wood-Werkman Reaction.—In 1935, Wood and Werk-

man[542] made the important observation that the carbon present in $CaCO_3$ was transformed into organic carbon in the propionic acid fermentation. It was subsequently shown that, corresponding to the disappearance of CO_2, an equivalent amount of succinic acid was produced.[543]

Substantiating evidence was provided by Carson *et al.*[56,57] Working with labeled CO_2, pyruvate, and washed cells of *P. pentosaceum*, they found the products of fermentation, succinic and propionic acids, both contained carboxyl-labeled carbon. In addition, there was evidence for the probable formation of radioactive oxalacetic acid.

These experiments furnished conclusive evidence for the heterotropic conversion of CO_2 via pyruvic acid to succinic acid. This conversion reaction has been designated as the Wood-Werkman reaction, and historically it represents the first experimental demonstration of the oft-suspected utilization of CO_2 in organic synthesis by heterotrophic bacteria.

The experiments, moreover, showed that succinic acid was indeed the precursor of propionic acid. This acid presumably arose from succinic in a reversible decarboxylation reaction, which in the presence of fluoride proceeded with greater vigor in the direction of decarboxylation, thus:

$$\underset{\text{pyruvic acid}}{COOH\cdot CO\cdot CH_3} + C^{11}O_2 \rightleftharpoons \underset{\text{oxalacetic acid}}{COOH\cdot CO\cdot CH_2\cdot C^{11}OOH} \quad \text{(R18)}$$

$$COOH\cdot CO\cdot CH_2\cdot C^{11}OOH + 4H \rightleftharpoons \underset{\text{succinic acid}}{COOH\cdot (CH_2)_2\cdot C^{11}OOH} + H_2O \quad \text{(R19)}$$

$$COOH\cdot (CH_2)_2\cdot C^{11}OOH \rightleftharpoons CO_2 + \underset{\text{propionic acid}}{CH_3\cdot CH_2\cdot C^{11}OOH} \quad \text{(R20)}$$

$$COOH\cdot (CH_2)_2\cdot C^{11}OOH \rightleftharpoons COOH\cdot CH_2\cdot CH_3 + C^{11}O_2 \quad \text{(R21)}$$

These are the reactions, now well substantiated, which account for the formation of propionic acid in this fermentation. It was thought at one time that propionic acid arose as a result of the reduction of lactic acid, thus:

$$CH_3\cdot CHOH\cdot COOH + 2H \rightarrow CH_3\cdot CH_2\cdot COOH + H_2O \quad \text{(R22)}$$

This reaction appears to occur in the formation of propionic acid from lactic acid by *Clostridium propionicum*. However, lactate is not generally a required intermediate in the formation of propionate.[16,128,229,458,531]

The Wood-Werkman reaction occurs widely in microorganisms. Fixation of CO_2 does not occur in the growth of *L. arabinosus* unless trace quantities of biotin are present. In the presence of biotin,

CO_2 is fixed and its carbon appears in aspartic acid. In the absence of biotin, aspartic acid is an essential growth factor for *L. arabinosus.*

Although the general outlines of the Wood-Werkman reaction are clear, there is ambiguity with regard to details.[175,377,383,397,495,496,544]

Transcarboxylation in Propionic Acid Fermentations.—Carboxylation at the 2-C of propionic acid rather than at the 3-C would be expected in reaction (R21). In animal tissues propionate is oxidized along a pathway involving first conversion to propionyl-coenzyme A (CoA)—a thioester which contains, as the discussion on p. 689 will show, an energy-rich thioester bond. Propionyl-CoA then undergoes an adenosine triphosphate dependent carboxylation not to succinyl-CoA but rather to methylmalonyl (isosuccinyl-CoA); and subsequently the carbon skeleton of methyl malonate is converted quantitatively to that of succinate which is then oxidized via the citric acid cycle.[121] Thus it is true that carboxylation occurs at the 2-C of propionic acid. A similar pathway is followed in propionic acid fermentations. Thus Swick and Wood working with cell-free extracts of *Propionibacterium shermanii* investigated the reactions leading from pyruvate to propionate and observed the following sequence, thus:[477]

$$\text{succinyl-CoA} \rightleftharpoons \text{methylmalonyl-CoA} \qquad \text{(R23)}$$

$$\text{methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxalacetate} \qquad \text{(R24)}$$

$$\text{oxalacetate} + 4\text{H} \overset{-\text{H}_2\text{O}}{\rightleftharpoons} \text{succinate} \qquad \text{(R25)}$$

$$\text{succinate} + \text{propionyl-CoA} \rightleftharpoons \text{succinyl-CoA} + \text{propionate} \qquad \text{(R26)}$$

The isomerization reaction (R23) is catalyzed by coenzyme B_{12}, an analog of vitamin B_{12}. The carboxylation of propionate is catalyzed by a biotin containing carboxylase.[385]

In the transcarboxylation reaction (R23), it is the thioester, rather than the carboxyl group of methylmalonyl-CoA which migrates. Eggerer *et al.* considered the following alternatives:[104]

$$\begin{array}{l} \text{HO}_2\text{C}\cdot\overset{\overset{\text{CH}_3}{|}}{\underset{\underset{*}{|}}{\text{CH}}}\cdot\text{COSCoA} \quad \left[\begin{array}{l} \xrightarrow{\text{COOH-migration}} \text{HO}_2\text{C}\cdot\text{CH}_2\cdot\overset{*}{\text{C}}\text{H}_2\cdot\text{COSCoA} \\ \xrightarrow[\text{COSCoA-migration}]{} \text{HO}_2\text{C}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COSCoA} \quad {}^{*} \end{array}\right. \end{array} \qquad \text{(R27)}$$

The asterisk indicates the labeled carbon atom.

Degradation of the resulting labeled succinyl-CoA, and study of the radioactivity of the degradation products supported the view that the thioester had migrated. Hegre *et al.* working with a purified methylmalonyl isomerase system and enzymatically synthesized [1-^{14}C] and [3-^{14}C] methylmalonyl·CoA reached the same conclusion.[192]

The Formation of Acetic and Carbonic Acids.—In the fermentation of lactate, the constancy of the ratio between the amounts of acetic and carbonic acids points to the existence of a common precursor, pyruvic acid. It is formed as a result of the oxidation of lactic acid, thus:

$$CH_3 \cdot CHOH \cdot COOH \rightleftharpoons CH_3 \cdot CO \cdot COOH + 2H \qquad (R28)$$

This reaction, catalyzed by lactic dehydrogenase, is the reverse of the reduction in glycolysis in which reduced coenzyme I is the hydrogen carrier. In order for the reaction to proceed in the direction of pyruvate, a hydrogen acceptor is necessary. This acceptor presumably is a flavoenzyme, succinic acid dehydrogenase, which converts oxalacetate reversibly to succinate. Oxalacetic acid, of course, is the product of the carboxylation reaction involving CO_2 and pyruvic acid.

With the formation of pyruvic acid, the conditions now exist for its decarboxylation coupled with the reduction of oxalacetic acid, thus:

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + H_2O \rightleftharpoons \underset{\text{acetic acid}}{CH_3 \cdot COOH} + CO_2 + 2H \qquad (R29)$$

$$\underset{\text{oxalacetic acid}}{COOH \cdot CO \cdot CH_2 \cdot COOH} + 4H \xrightleftharpoons{-H_2O} \underset{\text{succinic acid}}{COOH \cdot (CH_2)_2 \cdot COOH} \qquad (R30)$$

Two hydrogens are provided for the reduction of oxalacetic acid in the "dehydrogenative" decarboxylation of pyruvic acid (R29), and two are provided in the oxidation of lactic acid (R28). Thus an accounting has been made for two end products of fermentation, acetic and carbonic acids. Propionic acid is derived from succinic acid to the extent that succinic acid is decarboxylated; and thus the formation of the end products of lactic acid metabolism and the balance sheet with respect to carbon and to oxidation and reduction have been elucidated.

The Alcohol (Yeast) Fermentation

Investigation of the alcohol fermentation contributed greatly to the elucidation of the Meyerhof-Embden reaction pattern, a pattern which is now known to be shared by many bacterial types up to the point at which pyruvic acid is formed.

The alcohol fermentation is of more than theoretical interest in dairy technology. Whey is an excellent substrate for alcohol production, and alcohol is an important constituent of some cultured milks. The alcohol fermentation may be modified to produce potentially important lactose-fermenting yeasts.

The alcohol and the homolactic fermentations follow in part a com-

mon course, departing from it with the production of the important intermediate pyruvic acid. Possessing the enzyme carboxylase and the coenzyme diphosphothiamine,[317] yeast acts to decarboxylate pyruvic acid with the production of acetaldehyde and CO_2. The acetaldehyde is reduced forthwith in the presence of alcohol dehydrogenase and reduced coenzyme I (DPNH) to ethyl alcohol, thus:[144,262,273,415]

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} \xrightarrow{\text{pyruvic carboxylase}} \underset{\text{acetaldehyde}}{CH_3 \cdot CHO} + CO_2 \qquad \text{(R31)}$$

$$CH_3 \cdot CHO \underset{-2H}{\overset{+2H}{\rightleftharpoons}} \underset{\text{ethyl alcohol}}{CH_3 \cdot CH_2OH} \qquad \text{(R32)}$$

The Glycerol (Modified Alcohol) Fermentation

There is no reason to believe that lacteal sources are unsuitable for the microbiological synthesis of glycerol—a substance much in demand in situations such as wars produce. If, in the alcohol fermentation, the intermediate acetaldehyde is fixed with sulfite, the spared reducing substance (reduced DPN) acts on triose phosphate to produce glycerol. For every mole of acetaldehyde fixed, the production of one mole of glycerol becomes possible.[74,113,157,223,280,368,370-372,510,552]

In the presence of added alkali,[367,369,373] the normal course of the alcohol fermentation is also modified in the direction of glycerol formation. In a weak alkaline solution, the intermediate acetaldehyde undergoes an intermolecular Cannizaro oxidation-reduction, acetic acid and ethanol are formed, and reduced DPN again becomes available for the reduction of 3-phosphoglyceraldehyde to yield ultimately glycerol and inorganic phosphate. The overall reaction in the presence of sulfite is:

$$\underset{\text{glucose}}{C_6H_{12}O_6} + Na_2SO_3 + H_2O \rightarrow \underset{\text{glycerol}}{C_3H_8O_3} + \underset{\text{fixed aldehyde}}{C_2H_4O \cdot NaHSO_3} + NaHCO_3 \qquad \text{(R33)}$$

The overall reaction in the presence of alkali is:

$$2C_6H_{12}O_6 + H_2O \rightarrow CO_2 + \underset{\text{acetic acid}}{C_2H_4O_2} + \underset{\text{ethanol}}{C_2H_5OH} + \underset{\text{glycerol}}{2C_3H_8O_3} \qquad \text{(R34)}$$

A new approach to the problem of glycerol synthesis has been presented by Wallerstein and Stern[509] and Beloff and Stern.[23] They suggested the use of selective inhibitors of alcohol dehydrogenase or carboxylase in order to prevent either the reduction of acetaldehyde to alcohol or the formation of acetaldehyde from pyruvate; the fermentation would be directed toward the formation of glycerol, and the use of large quantities of fixing or trapping reagent would be avoided.

The Oxidative Decarboxylation of Pyruvic Acid

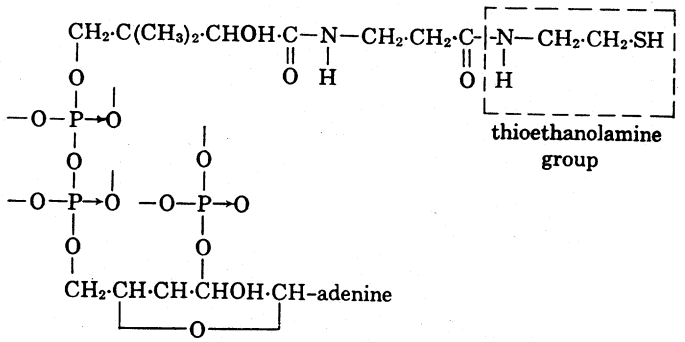
Pyruvic acid is clearly an intermediate whose existence makes possible a multiplicity of parallel reactions. With the formation of this acid, the main pathway in the dissimilation of glucose begins to branch. Further branching, even greater in its multiplicity, occurs upon the oxidative decarboxylation of pyruvic acid. An active two-carbon fragment is formed, and this compound enters into a series of parallel reactions which account for many of the products of fermentation. Before considering other fermentation types, it would be profitable to consider the formation of this active intermediate.

In fermentations with heterolactic bacteria, acetic acid and CO_2 are commonly produced in appreciable quantities. With *L. delbrückii*, homofermentative under anaerobic conditions, pyruvic acid under aerobic conditions is converted into acetic acid and CO_2, thus:

$$CH_3 \cdot CO \cdot COOH + {}^1/_2\, O_2 \rightarrow CH_3 \cdot COOH + CO_2 \qquad \text{(R35)}$$

Anaerobically, pyruvic acid may undergo dismutation to form lactic, acetic, and carbonic acids. Lipmann,[309] working with an enzyme solution prepared from *L. delbrückii*, showed that phosphate was essential for the oxidative decarboxylation. This led eventually to the isolation of acetyl phosphate as a product of the oxidation. Later the same conditions were found to hold in the formation of acetyl phosphate in various anaerobic and facultative anaerobic organisms, such as *E. coli*, *Clostridium butylicum*, *Clostridium saccharobutyricum*, *Clostridium sporogenes*, *Aerobacter aerogenes*, and *S. faecalis*.

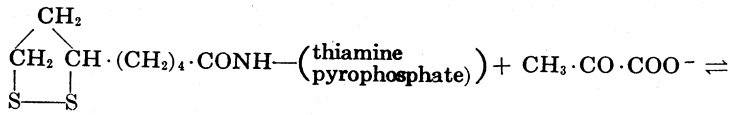
Acetyl phosphate can serve as an acetyl or acetate donor provided that an activator known as coenzyme A is present.[311,313] The structure of the compound appears to be:[90]



It contains adenylic acid and a pyrophosphate bridge, cross-linking the adenylic 5′-position with the 4′-position of pantothenic acid. The pantothenic acid is peptide-linked via its terminal carboxyl

group with thioethanolamine, and this derived moiety is known as pantetheine. Pantetheine and its oxidized disulfide form, pantethine are growth factors required by *L. bulgaricus* and *Lactobacillus acidophilus*. The bacterial (*Proteus morganii*) requirement for pantetheine is closely related to its involvement in coenzyme A.[101,204,381]

The oxidative decarboxylation of pyruvic acid is now considered to consist of four stages.[257] In the first stage, pyruvate reacts (see R36) with lipothiamidepyrophosphate (LTPP), which is a complex of lipoic acid and thiamine pyrophosphate (TPP):[386,419,420]



$$CH_3\cdot COS—CH_2\cdot CH_2\cdot CHS^-\cdot(CH_2)_4\cdot CONH—(TPP) + CO_2 \qquad (R36)$$

In the second stage, the acetyl group is transferred from the acetyl lipothiamidepyrophosphate complex to coenzyme A. This reaction is mediated by a transacetylase, thus:

$$CoA—SH + CH_3\cdot CO—S—(LTPP)—S^- \rightleftharpoons CoA—SOC\cdot CH_3 + HS—(LTPP)—S^- \qquad (R37)$$

In this stage also, LTPP is regenerated. Hydrogen from reduced lipothiamidepyrophosphate is transferred to DPN_{ox} (oxidized DPN). Hydrogen from DPN_{red} (reduced DPN) is then transferred to O_2 via a flavoprotein enzyme to form H_2O_2, as in the oxidative decarboxylation of pyruvate by *L. delbrückii*. Alternately, the hydrogen may be transferred to pyruvate via lactic dehydrogenase, as in the anaerobic oxidative decarboxylation of pyruvic acid, and lactic acid is formed.

In the third stage acetyl phosphate is formed and in the fourth and final stage, acetate and ATP, thus:

$$CH_3\cdot COS \sim (CoA) + H_3PO_4 \rightleftharpoons CH_3\cdot CO_2 \sim H_2PO_3 + CoA—SH \qquad (R38)$$

$$CH_3\cdot CO_2 \sim H_2PO_3 + ADP^- \rightleftharpoons ATP^- + CH_3COO^- + H^+ \qquad (R39)$$

Reaction (R38) is mediated by phosphotransacetylase.

Fermentations of the E. Coli Type

Escherichia is one genus of the tribe *Escherichieae*. This tribe also includes *Aerobacter* and *Klebsiella*. Members of *Escherichieae*, if acetylmethylcarbinol is not produced and if the methyl red test is positive are said to belong to the genus *Escherichia*. If acetylmethylcarbinol is produced, if the methyl red test is negative, and if citrates may be used as the sole source of carbon, members of *Escherichieae* are said to belong to the genus *Aerobacter*. Members of the

genus *Escherichia* ferment many substrates including lactose with acid and gas production.[151] Carbon dioxide and hydrogen are produced from glucose in approximately equal volumes. The coliforms when grown in milk and milk products may bring about gassiness, early gas formation in cheese for example, in addition to flavor defects.

Quantitatively, the end products of fermentations with *E. coli* are apt to be quite variable and are influenced by pH (see Table 98). The percentage of alcohol produced appears to be invariant with respect to changes in pH.[488] The formic acid represents free acid and that which is decomposed according to the equation:

$$HCOOH \rightleftharpoons H_2 + CO_2 \qquad \text{(R40)}$$

The production of lactic acid diminishes, and that of acetic and formic acids increases, as pH increases. This points to the likelihood that pyruvic acid is a common source for these acids but not for ethanol.

In the presence of $CaCO_3$, it is generally believed that *E. coli* will ferment glucose to yield one part carbon dioxide, one part hydrogen, one part acetic acid, one part alcohol, two parts lactic acid, and one-half part succinic acid. Some of the *Enterobacteriaceae*, such as *Salmonella typhosa*, yield little H_2 gas and correspondingly large quantities of formic acid.

The Formic Acid Fermentation.—Formic acid production is a characteristic of *E. coli*. The pathway to pyruvic acid has been studied quite extensively in connection with fermentations by *E. coli*. With respect to both intermediate products and characteristic enzyme systems, the composition of *E. coli* cell juices parallels in a large measure that of yeast juice.

Harden deduced in 1901 that hydrogen is derived from formic acid (see Tikka[488]). With some kinds of bacteria, formic acid is produced but hydrogen is not. With mixed cultures, formic acid is produced from pyruvic acid, thus:[365]

$$CH_3 \cdot CO \cdot COOH + H_2O \rightarrow \underset{\text{acetic acid}}{CH_3 \cdot COOH} + \underset{\text{formic acid}}{HCOOH} \qquad \text{(R41)}$$

TABLE 98

FERMENTATION OF GLUCOSE BY *E. coli* AT DIFFERENT pH LEVELS[a]

Glucose Gm.	Initial H-ion Conc., pH	Lactic Acid Mg.	Lactic Acid %	Acetic Acid Mg.	Acetic Acid %	Formic Acid Mg.	Formic Acid %	Ethanol Mg.	Ethanol %	H_2 Ml
4.000	7.1	815	20.4	724	18.1	994	16.2	645	21.0	45
4.000	7.1	860	21.5	812	20.3	736	12.0	676	22.0	46
2.000	6.4	926	46.3	90	4.5	85	2.8	325	21.1	181
2.000	6.4	816	40.8	120	6.0	113	3.7	296	19.3	146
2.000	7.4	82	4.1	588	29.4	622	20.2	340	22.1	29
2.000	7.6	54	2.7	683	34.1	822	26.8	325	21.1	37

[a] Data from a publication by Tikka.[488]

The reaction follows a detailed course. Jones *et al.*[233] studied the reaction with labeled $CH_3 \cdot C^{14}OOH$ and labeled pyrophosphate. Their findings may be summarized in the following series of reactions:

$$CH_3 \cdot CO \cdot COOH + HS(CoA) \rightleftharpoons CH_3 \cdot COS(CoA) + HCOOH \quad (R42)$$

$$ad\text{—}P \sim PP + enz \cdot OH \rightleftharpoons enz \sim P\text{—}ad + pyrosphosphate \quad (R43)$$

$$enz \sim P\text{—}ad + HS(CoA) \rightleftharpoons enz \sim S(CoA) + adenylic\ acid \quad (R44)$$

$$CH_3 \cdot COOH + enz \sim S(CoA) \rightleftharpoons CH_3 \cdot CO \sim S(CoA) + enz \cdot OH \quad (R45)$$

$$CH_3 \cdot CO \sim S(CoA) + phosphate \rightleftharpoons CH_3 \cdot CO(O \sim PO_3H_2) + HS(CoA) \quad (R46)$$

AD-P $\sim$ PP (see R43) is bound by the apoenzyme, and modifies it, with the release of pyrophosphate and the formation of a protein-adenylic acid complex. The overall reaction leading from pyruvic acid to formic and acetic acids is:

$$CH_3 \cdot COOH + HCOOH + ATP \rightleftharpoons \underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + pyrophosphate + adenylic\ acid \quad (R47)$$

The Formation of H_2.—The reversibility of the conversion of formic acid to H_2 and CO_2 (see R40) has been demonstrated with *E. coli* extracts and radioactive $NaHC^{13}O_3$. There is a clear implication that molecular hydrogen can be utilized by *E. coli* to effect the reduction of carbonic acid. The question arises: How is this brought about?

Stephenson and Stickland[473] believe that three enzymes are required in hydrogen formation. Two of the three are necessary to account for the following reactions, thus:

$$HCOO^- + H_2O \overset{\text{dehydrogenase}}{\rightleftharpoons} HCO_3^- + 2H^+ + 2e^- \quad (R48)$$

$$2H^+ + 2e^- \overset{\text{hydrogenase}}{\rightleftharpoons} H_2 \quad (R49)$$

Hydrogen is not always produced in the presence of both dehydrogenase and hydrogenase. The result is attributed to the absence of an intermediate electron carrier named "hydrogenlyase."[473] "Hydrogenlyase," although it does not develop in suspensions of *E. coli* in glucose, does develop in broth suspensions containing one per cent formate. Synthesis of "hydrogenlyase" is observed after one hour. Growth is not required.

The Formation of Ethanol.—It is generally agreed that the ethanol produced with *E. coli* does not arise in the same manner as that produced with yeast. Dawes and Foster[89] have proposed the following scheme:

$$pyruvic\ acid \rightarrow acetyl\ CoA \rightarrow acetaldehyde \rightarrow ethanol \quad (R50)$$

When *E. coli* fermented glucose, the concentration of ethanol increased during the first 60 min. and thereafter decreased. Acetaldehyde was identified as a true intermediate. With the use of cell suspensions of a pantothenate-requiring mutant, it was shown that CoA was a cofactor in ethanol production. Ethanol dehydrogenase and acetaldehyde dehydrogenase were prepared in partially purified forms.

The Formation of Lactic Acid.—The evidence is strongly in favor of parallel metabolic pathways for homolactics and *E. coli* in the production of lactic acid from glucose. The presence of lactic dehydrogenase in *E. coli* has been studied by Quastel and Whetham.[413]

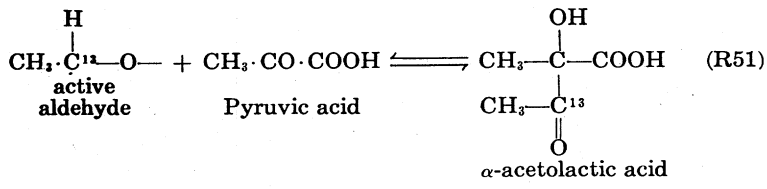
The Aerobacter Type Fermentation

Biochemically, the genus *Aerobacter* is differentiated from *Escherichia* in this respect, among others, that whereas *Escherichia* produces CO_2 and H_2 in equimolar ratios, *Aerobacter* produces from 5 to 8 times more CO_2. A concomitant of this is the formation of acetylmethylcarbinol in the *Aerobacter* but not in the *Escherichia* type fermentation.[422]

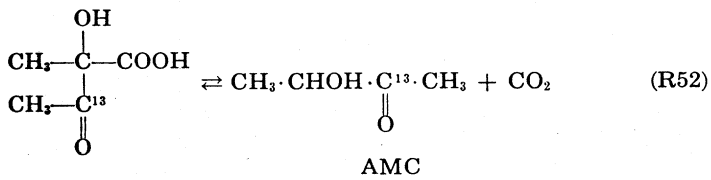
Species of the genus *Aerobacter* are facultative anaerobes. *Aerobacter aerogenes* is normally found on grains and plants and to a varying degree in the intestinal canal of man and animals. *A. cloacae* is found in human and animal feces as well as in sewage, soil, and water. The differentiation of *Aerobacter* from *Escherichia* permits differentiation of sources of contamination, whether animal or soil, of a milk supply.

The Formation of Acetylmethylcarbinol.—Acetylmethylcarbinol (AMC) may be obtained by reduction of biacetyl. The flavor of butter has been attributed to biacetyl. Its production in butter-making has been ascribed to the action on citrate of *L. citrovorum* and *L. dextranicum*, two bacteria unrelated to *Aerobacter* species. Nevertheless, the production of biacetyl in the intermediary metabolism of *Aerobacter* species bears on the general question of its origin. It should be borne in mind in this connection that *Aerobacter*, like the aforementioned *Leuconostoc* species, can utilize an exogenous pyruvate source as a sole source of carbon.[50]

Slade and Werkman[461] explained the action of *A. cloacae* (*Aerobacter indologenes*) cells on carboxy-carbon labeled acetate and pyruvate, thus:



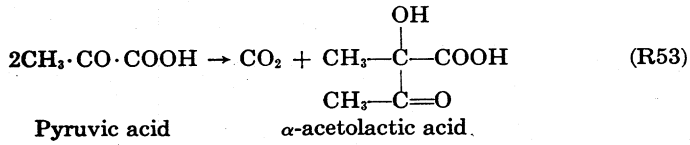
(R51)



(R52)

The active aldehyde would be formed from acetate through a reversal of the reaction leading from pyruvate to acetate. Two enzymes are required—one to catalyze the condensation to α-acetolactic acid and the other to foster the decarboxylation. The labeled molecule in the first of the two reactions (R51) is either acetaldehyde or a closely related derivative formed by the reduction of labeled acetate.

With *Aerobacter* species not utilizing acetaldehyde, the following intermediate reaction occurs, thus:



(R53)

Juni[234] found enzymes both for reactions (R51) and (R52) in cell-free preparations from *A. aerogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Serratia marcescens*. The enzyme apparatus of *E. coli* containing only α-acetolactic decarboxylase is an exception to the rule that the enzymes mentioned above always occur together.

Cell suspensions of *S. faecalis* promoted oxidation of pyruvate to acetate and CO_2, or anaerobically to lactate, acetate and CO_2, whereas cell-free extracts catalyzed the dismutation to AMC and CO_2 at a pH optimum of 6.1.[100]

The detailed pathway to AMC is probably not universal. Animal tissues can metabolize acetaldehyde to AMC in the absence of pyruvate; yeast can metabolize acetaldehyde only in the presence of pyruvate; and finally *Aerobacter* species cannot utilize acetaldehyde.

Martius and Lynen[330] have proposed the following mechanism to account for the requirement of phosphate in *Aerobacter* metabolism and for the utilization of acetaldehyde by *Bacillus polymyxa:*

$$CH_3\cdot COO \sim H_2PO_3 + CH_3\cdot \underset{\underset{H}{|}}{C} = O \rightarrow CH_3\cdot CO\cdot CO\cdot CH_3 + H_3PO_4 \quad (R54)$$

acetylphosphate acetaldehyde biacetyl

Biacetyl rather than AMC is the primary product. In succeeding steps, biacetyl is reduced to AMC, and AMC to 2,3-butylene glycol.

In the so-called AMC fermentation very little AMC and biacetyl are actually found; 2,3-butylene glycol, the reduction product, accumulates.

The oxidation of 2,3-butylene glycol to biacetyl, nonenzymatically, proceeds in alkaline solution. Biacetyl condenses with peptone constituents or α-naphthol and creatine, to yield the bright pink product of the Voges-Proskauer reaction which is positive for *Aerobacter* and characteristically differentiates *Aerobacter* from *Escherichia*.

The products of typical *Aerobacter* and *Escherichia* fermentations are compared in Table 99.[423]

Industrially, studies on the 2,3-butylene glycol (2,3-butanediol) fermentation have been centered around the two organisms, *A. aerogenes*, and *B. polymyxa*.[27,28,152,364] The results may bear on possible uses for whey.

TABLE 99

PRODUCTS OF TYPICAL *Aerobacter* AND *Escherichia*[a] FERMENTATIONS

Product	Yield, Moles per 100 Moles Glucose	
	A. indologenes	*E. coli*
CO_2	172	44
H_2	36	43
Formic acid	18	2
Acetic acid	0.5	44
Ethanol	70	42
Lactic acid	3	84
Succinic acid	0	29
2,3-Butylene glycol	66.5	

[a] Data from Reynolds and Werkman.[423]

Butyric Acid and Related Fermentations

Of microorganisms producing butyric acid, the *Clostridium* species are technologically the most important, giving rise in the butyl alcohol-acetone fermentation to valuable industrial solvents and the vitamin, riboflavin. In dairy technology the *Clostridium* species give rise to late gassing in cheese and to off-flavors.

The *Clostridium* species are gram positive, spore-forming rods, either strictly anaerobic or microaerophilic. The members of the

genus differ in their fermentations. *Clostridium butyricum*, synonymous with *C. saccharobutyricum*, produces typically the following from 100 moles fermented glucose: 233 moles hydrogen, 196 moles carbon dioxide, 43 moles acetic, and 75 moles butyric acid. Other species of the genus producing large quantities of butyric acid are: *Clostridium amylobacter*, *Clostridium pasteurianum*, and *Clostridium lactoacetophilum*, which, as the name implies, is active toward lactate in the presence of acetate.

Closely related to the butyric is the butylic fermentation. Butylic clostridia—*C. butylicum*, *Clostridium acetobutylicum*, and others—are able to carry the butyric fermentation to a more advanced stage, leading to the production of butanol and other volatile solvents.

The Butyric Acid Fermentation.—In the formation of butyric acid, C_3 compounds may be utilized (glycerol by *C. acetobutylicum*, pyruvate by *C. butylicum*, and lactate by *C. lactoacetophilum*). Consequently it would appear that butyric acid formation is not a direct result of the splitting of a hexose into C_4 and C_2 components.

In the light of the demonstrated importance of coenzyme A in the oxidation of butyric acid, Barker[14] formulated the following series of reactions:

$$2\ CH_3\cdot CO\cdot COO^- + 2CoA \underset{+2CO_2}{\overset{-2CO_2}{\rightleftharpoons}} 2\ \underset{\text{acetyl-CoA}}{CH_3\cdot CO^-CoA} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \underset{\text{acetoacetyl-CoA}}{CH_3\cdot CO\cdot CH_2\cdot CO—CoA} \quad \text{(R55)}$$

$$CH_3\cdot CO\cdot CH_2\cdot CO—CoA \underset{-2H}{\overset{+2H}{\rightleftharpoons}} \underset{\text{vinylacetyl-CoA}}{CH_2{=}CH\cdot CH_2\cdot CO—CoA} \text{ or}$$

$$\underset{\text{crotonyl-CoA}}{CH_3\cdot CH{=}CH\cdot CO—CoA} + H_2O \quad \text{(R56)}$$

$$\text{vinylacetyl—CoA} \underset{-2H}{\overset{+2H}{\rightleftharpoons}} \underset{\text{butyryl—CoA}}{CH_3\cdot CH_2\cdot CH_2\cdot CO—CoA} \quad \text{(R57)}$$

$$\text{butyryl—CoA} \rightleftharpoons \text{butyrate} + \text{CoA} \quad \text{(R57a)}$$

Attempts to demonstrate the conversion of pyruvate to butyrate met with difficulties. *C. butylicum* converts pyruvate into acetate, CO_2 and H_2 at an optimum pH of 5.0. With cell-free extracts, in the presence of phosphate, acetyl phosphate, CO_2, and H_2 are formed.

The mechanism postulated for the conversion of acetate to butyrate also explains the conversion of lactate to butyrate by *C. lactoacetophilum*. Acetate is required. The condensation again involves CoA, and is mediated by an independent condensing apoenzyme. One mole of added acetate is consumed per mole of butyric acid formed.

In the formation of butyric acid from lactic acid by *Butyribacterium rettgeri*, CO_2 serves as a precursor of additional acetate, and is required, thus:

$$CH_3 \cdot CHOH \cdot COOH + CO_2 + 4H \rightarrow 2CH_3 \cdot COOH + H_2O \quad (R58)$$

Energetically, the reaction involving the utilization of CO_2 in the formation of a -C-C-bond proceeds by virtue of the simultaneous oxidation of lactate to yield CO_2.[15]

The butyric fermentation and the conversion of pyruvate to acetate by *C. acetobutylicum* are inhibited by CO.[241,272] Inactivation of an iron-containing protein complex appears to be involved.[512]

The Butanol Fermentation.—In the commercial production of acetone and butanol by the action of *C. acetobutylicum* on corn meal, the following products are formed in the early stages of fermentation: lactic and acetic acids, CO_2 and H_2. A so-called "break" is reached after 13–17 hr. in an active fermentation, coinciding with the development of maximum titratable acidity. Then the acid titer decreases rapidly, and at the same time the production of volatile solvents begins and increases rapidly. The acids are precursors of the solvents.

Studies by Koepsell *et al.*[251] with cell-free extracts of *C. butylicum*, and by Stadtman and Barker[470] with *Clostridium kluyveri* may be summarized, thus:

$$CH_3 \cdot COOPO_3^- + CH_3 \cdot (CH_2)_2 \cdot COO^- \rightleftharpoons CH_3COO^- + CH_3 \cdot (CH_2)_2 \cdot COOPO_3^- \quad (R59)$$

$$C_3H_7 \cdot COOPO_3^- + 2\,H_2 \rightarrow C_3H_7 \cdot CH_2OH + HPO_4^- \quad (R60)$$

The yields were as high as 77% of theoretical. In view of later findings it would seem that butyryl-CoA rather than butyryl phosphate is the direct precursor.

Eliasburg,[106] comparing the effects in the butanol fermentation of hydrogen at atmospheric and at 20 atmospheres pressure, observed that the increase in pressure brought about a decrease in the yield of butyric acid from 30 to 15%, and correspondingly an increase in yield of butanol from 0.3 to 8%.

The Acetone and Isopropanol Fermentation.—Addition of acetate to an active maize fermentation liquor results in increased yields of acetone.[421] With resting cell suspensions of *C. acetobutylicum*, two moles of acetate yield one mole of acetone and one mole of CO_2. The reaction proceeds by way of acetoacetic acid, and the energy requirement for the condensation of acetic acid is met in the simultaneous decomposition of glucose or pyruvate.[87] Acetoacetic decarboxylase, prepared from suspensions of *C. acetobutyli-*

cum, has properties resembling somewhat those of riboflavin phosphate.[88] The formation of acetone is adequately represented by the following reactions:

$$\underset{\text{acetic acid}}{2\ CH_3\cdot COOH} \rightleftharpoons \underset{\text{acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} + H_2O \qquad \text{(R61)}$$

$$CH_3\cdot CO\cdot CH_2\cdot COOH \rightleftharpoons \underset{\text{acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2 \qquad \text{(R62)}$$

In the isopropanol fermentation by *C. butylicum*, the addition of labeled acetone results in the formation of labeled isopropanol. Adding two moles of acetic acid results in the formation of one mole of isopropanol and one mole of CO_2. Clearly, isopropanol must result from the reduction of acetone.

Formation of ethanol, to the extent of 2–3% of glucose fermented, always accompanies the formation of butanol. Ethanol derived from fermentations in which labeled acetate is present in the media always contains labeled carbon, and it may be presumed, therefore, that ethanol derives from acetate via coenzyme A and an appropriate dehydrogenase system.

Respiratory Processes

The terminal products of respiration are CO_2 and H_2O. In fermentation the ultimate acceptor of electrons and protons is some organic molecule, for example DPN, and in respiration it is oxygen. Calculations based on available redox potentials (see Table 100) show that for each two electron transfer, the free energy change accompanying respiration exceeds by 53.4 kcal./mole of product that accompanying fermentation. Respiration therefore sets free larger stores of energy than does fermentative oxidation.

What mechanism underlies the transfer of hydrogen to oxygen? There is no unique mechanism, yet the number of mechanisms is quite limited. Transfer occurs in a sequence of steps in which electrons and protons move through a series of acceptors to molecular oxygen. Organic substrates under conditions of culture yield electrons and protons to appropriate enzyme systems known as dehydrogenases. These can then effect the transfer of their acquired complement of electrons and protons either to the so-called cytochrome system or to coenzymes, the latter of which are bound with some measure of firmness to the protein moiety of dehydrogenases. Reduced coenzymes can then transfer electrons and protons to flavoprotein enzymes; and thus in regaining their oxidized state they may again accept hydrogen. The detailed mechanism underlying the transfer of hydrogen from one coenzyme to another is obscure. A number of

TABLE 100

OXIDATION-REDUCTION POTENTIALS[a]

Oxidation-Reduction System		Temp., °C.	pH	E_h Volt
Oxidized Compound	Reduced Compound			
Coenzyme I	Reduced coenzyme	30	7.0	−0.282
Riboflavin	Leuco-riboflavin	20	7.0	−0.186
Heme, ferri	Heme, ferro	30	8.18	−0.188
Pyruvate	Lactate	35	7.01	−0.180
Maleate	Succinate	37	7.0	−0.094
Ferric ion	Iron	25	0.0	−0.36
Cytochrome b, Fe^{3+}	Cytochrome b, Fe^{2+}	20	7.4	−0.04
α-Ketoglutarate	Glutamate		7.0	−0.030 calc.
Methylene blue	Leuco-methylene blue		7.0	+0.011
Cytochrome a, Fe^{3+}	Cytochrome, Fe^{2+}	20	7.4	+0.262
Oxygen	Water	25	7.0	+0.815
Iodine	Iodide	25	0.0	+0.5345
Oxygen	Hydrogen peroxide	25	0.0	+0.682
Oleate	Palmitate		7.0	+0.025
Fumarate	Succinate		7.0	0.00
Resazurin	Hydroresorufin		7.0	+0.051
Acetaldehyde	Ethanol	20	7.0	−0.200
Cystine	Cysteine	25	7.0	−0.14
Gluconate	Glucose		7.0	−0.45
Carbon dioxide	Formate	30	7.0	−0.42
Hydrogen ion	Hydrogen	All temps	0.00	0.000

[a] From Anderson and Plaut.[5]

schemes have been postulated recently showing how energy-rich bonds can be formed with reduced DPN, flavin-adenine-dinucleotide and ferricytochrome a_3.[139,150]

The Flavoproteins

The flavoproteins constitute a system of important oxidases and dehydrogenases containing as part of their molecular structure either riboflavin mononucleotide (FMN) or more commonly flavin-adenine dinucleotide (FAD).

The view long held that flavoprotein enzymes are limited to the role of terminal oxidases has now been abandoned. Some flavoproteins contain metal which participates as an essential link in an electron transport system. Thus, xanthine oxidase, familiar to dairy chemists because it is associated with the fat globule membrane in substantial amounts, is a metalloflavoprotein. Evidence that it is an iron-containing molybdo-flavoprotein has come from many sources.[65,96,426,489] Whitely and Ordal investigated the role of xanthine oxidase and hydrogenase, both from *Micrococcus lactilyticus*, in the transport sequence associated with the following reaction:[532]

$$\text{hypoxanthine} \rightleftharpoons \text{xanthine} + H_2 \qquad \text{(R63)}$$

Electron transport proceeds according to the sequence shown below:

$$\text{hypoxanthine} \rightarrow \underset{\text{xanthine oxidase (an iron-containing molybdo-flavoprotein)}}{\overset{X}{\rightarrow}} \underset{\text{hydrogenase (an iron-containing molybdo-flavoprotein)}}{\rightarrow} \text{hydrogen} \qquad \text{(R64)}$$

X is a heat-labile coupling compound, probably one containing iron.

Other metalloflavoproteins which have been studied during the past decade are the following: reduced DPN-cytochrome c-reductase which contains 4 Fe atoms for each flavin molecule,[326] butyryl-coenzyme A dehydrogenase which contains Cu,[145] aldehyde oxidase and reduced DPN-nitrate-reductase which contain molybdenum,[375] and fumaric hydrogenase and acyl-CoA dehydrogenase which contain Fe.[80]

Xanthine oxidase causes a slow reduction of methylene blue in "sterile" raw milk. The reduction time may be shortened appreciably by the introduction of xanthine, hypoxanthine, or other suitable substrates. Thermal inactivation of the enzyme requires temperatures as high as 85°C. Milks heated at this or higher temperatures suffer significant losses in xanthine oxidase activity.[145,216,400]

Among other oxidases one finds glucose oxidase, 6-phosphoglucose oxidase, cytochrome c oxidase, nonspecific D- and L-amino oxidases,[29] and the specific D-aspartic oxidase. The following two reac-

tions are mediated with impure and pure preparations respectively of L- and D-amino oxidases, thus:

$$R\cdot CH(NH_2)\cdot COOH + {}^1/_2O_2 \rightarrow R\cdot CO\cdot COOH + NH_3 \qquad (R65)$$

Pure preparations behave differently, thus:

$$R\cdot CH(NH_2)\cdot COOH + O_2 + H_2O \rightarrow R\cdot CO\cdot COOH + NH_3 + H_2O_2 \qquad (R66)$$

The oxidative decarboxylation of amino acids, leading in microbial metabolism to the formation of flavor-producing aldehydes and ketones, may be of importance in the manufacture of mold-ripened cheeses such as Roquefort, and in the manufacture of cultured milks such as yoghurt.

Less widespread among microorganisms than the amino acid oxidases are the diamino-oxidases, which catalyze the oxidation of diamines to ammonia, aldehydes, and hydrogen peroxide. These oxidases are apparently flavoproteins possessing a prosthetic group of the FAD type.

The Respiration of Lactic Acid Bacteria.—Bertho and Glück[25A] showed that catalase-free, facultative anaerobic, homolactic bacteria underwent a forced respiration in the presence of oxygen to yield hydrogen peroxide. Later, Warburg and Christian[514] proved that, in Bertho's experiments, the presence of Warburg's yellow ferment was responsible for the entire fermentation. In the respiration of *L. delbrückii* and *L. acidophilus* with the substrate glucose, one-half of the oxygen consumed is utilized in CO_2 production, the other half appearing in hydrogen peroxide. In later stages, the fermentation product, pyruvic acid, reacts with hydrogen peroxide, and the ratio between CO_2 formed and O_2 consumed increases. The respiration of homolactics proceeds through the intervention of flavoprotein oxidases, foremost among which is 6-phosphoglucose oxidase. This phenomenon manifests itself in such a way that the color of a suspension of certain bacteria can be transformed from white to yellow by the admission of air, and alternately it may be transformed from yellow to white by the exclusion of air.

The respiration is unphysiologic, and unless the medium contains some means of dissipating peroxide and preventing its accumulation, inhibition occurs. The "viridans" group of streptococci, and *L. bulgaricus* and *L. acidophilus*, produce a green pigment, a peroxidation product of hemoglobin, when grown on blood agar.[292,337]

Added peroxide inhibits the growth of the aerobes *Pasteurella pestis* and *Pasteurella septicum*, and hematin reverses the effect. The function of hematin, blood or catalase is to reverse the inhibition brought about by the product of respiration, hydrogen peroxide.

The accumulation of H_2O_2 accounts for the observation that strong aeration initially inhibits the growth of starter organisms. Although lactic acid organisms do not as a rule contain catalase, they do contain peroxidases which tend to dissipate the initial accumulation of H_2O_2. Thus Dolin has shown that *S. faecalis* in oxidizing reduced DPN produces H_2O_2 which in turn is reduced in the presence of a flavin-containing peroxidase.[99] Hydrogen peroxide produced by lactic acid bacteria strongly inhibits the growth of anaerobes.[530]

The uniqueness of the flavoproteins in the transfer of electrons has been postulated to reside in the structure of the flavin moiety which conduces to formation of semiquinones, i.e., intermediate free radicals.[349]

The Cytochrome System

Dehydrogenases may transfer electrons and protons to the cytochrome system or they may transfer hydrogen to coenzymes. Reduced coenzymes are reoxidized in respiration by way of flavoenzymes, which in turn are reoxidized either directly by oxygen, or indirectly by way of the cytochrome system. The cytochrome system, comprising the cytochromes and cytochrome oxidase, is extremely important.

In the dehydrogenation of succinate to fumarate and water, the following sequence is observed in the transfer of single electrons:

$$\text{succinate} \xrightarrow{e \mp H^+} Fe^{+++}\text{-cytochrome b} \xrightarrow{e \mp H^+} \text{Slater's factor} \xrightarrow{e \mp H^+} Fe^{+++}\text{-cytochrome c} \xrightarrow{e \mp H^+} Fe^{+++}\text{-cytochrome a} \xrightarrow{e \mp H^+} Fe^{+++}\text{-cytochrome oxidase-}O_2. \quad \text{(R67)}$$

This cytochrome system is absent in anaerobes such as the clostridia, and in homolactic bacteria. It is present in yeasts, *B. subtilis*, and *Pseudomonas* species, among others.

E. coli appears to contain modified cytochromes and a modified cytochrome oxidase, distinguished by their absorption bands.[462]

The Dehydrogenases

The ordinary dehydrogenases, with some exceptions, transfer two hydrogens and two electrons from a substrate to a codehydrogenase, usually coenzyme I or coenzyme II. One exception is succinic dehydrogenase, which, according to some evidence, is a flavoprotein. A second exception is the formic acid dehydrogenase of bacteria. This bacterial dehydrogenase seems to require cytochrome b; and of the same character are yeast and bacterial lactic acid dehydrogenases.

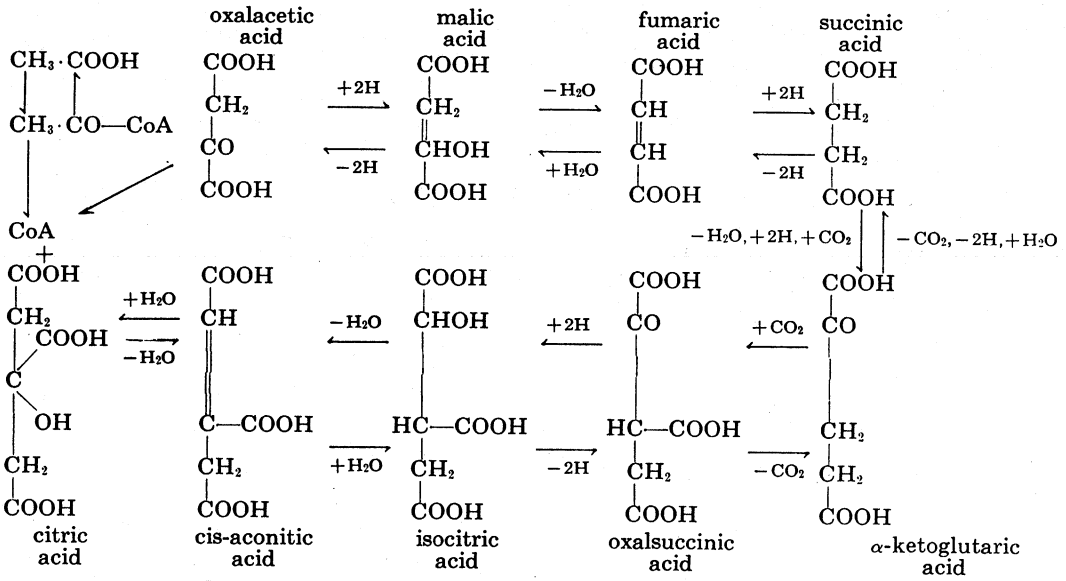
A third exception is the pyruvic acid oxidizing system, which contains lipothiamidepyrophosphate among its ensemblage of coenzymes as the primary coenzyme involved in the oxidation of pyruvic acid.

Besides biological acceptors, there are numerous nonbiological ones, such as methylene blue and resazurin. They furnish a basis for various tests designed to characterize the quality of milk and milk products.

The Krebs Cycle

In the oxidative decarboxylation of pyruvic acid, acetyl-CoA is one of the reaction products. In terminal respiration this compound together with oxalacetate initiates a series of enzymatically mediated reactions which proceed cyclically with the regeneration of oxalacetic acid, the consumption of two molecules of water and the formation of two molecules of carbon dioxide. Thus the oxidation is executed in a stepwise fashion such that the energy released is made available to the cell, thereby satisfying its energy requirements for growth, reproduction, movement, and other activities concerned with survival. The cycle of reactions known as the "Krebs" or citric acid cycle takes place in animal tissues.[267,268] In the metabolism of microorganisms, the concept of the operation of such a cycle, although the subject of much investigation and controversy, has only recently gained acceptance.[478,528] The Krebs cycle of intermediates is shown on p. 704.

The Krebs Cycle in Amino Acid Synthesis.—An interesting recent development is the discovery of the importance of the Krebs cycle in the metabolism of amino acids. In oxidative deaminations mediated by α-amino acid oxidases and dehydrogenases, α-oxoacids are formed together with NH_3. Transamination reactions can effect exchange between amino and ketonic groups. The Krebs cycle contains three α-oxoacids (oxalacetic, oxalsuccinic, and α-ketoglutaric) and therefore it can accommodate the deamination products of certain α-amino acids, or conversely it can serve as a source of α-amino acids and subsequently proteins. Two patterns of C^{14} distribution in amino acids of *E. coli* have been found.[1,338,433] These acids were derived from one of the following: $C^{14}O_2$, $C^{14}H_3COOH$, $CH_3C^{14}OOH$, aspartic acid, and glutamic acid both labeled uniformly and 1-C^{14} labeled glutamic acid. Grown in the presence of C^{12}-glucose and $C^{14}O_2$, *E. coli* yielded cellular proteins which when degraded showed: (1) that 20–40% of aspartic acid activity was located in C-1 and the balance in C-4; and (2) that more than 97% of glutamic acid activity resided in C-1. When $CH_3C^{14}OOH$ was used,



AN EXAMPLE OF THE KREB CYCLE.

analysis of the degraded proteins showed that 80 + % of the activity resided in C-5, and 5 + % in C-1, of glutamic acid. The values were those which would obtain on the assumption of the operation of a tricarboxylic acid cycle. Specific activity measurements and competition experiments showed that biosynthetic relationships existed among most of the amino acids. It was suggested that two separate acetate fixation mechanisms were in operation in amino acid synthesis—one in which leucine was a product, and a second in which glutamic acid or a substance in equilibrium with it, α-ketoglutaric acid, was an early product. With respect to the two families of amino acids, the members of each of which followed a definite and specific radioactivity pattern based on the specific activity of either glutamic or aspartic acid, the following genesis was postulated:

glutamic acid → glutamic-γ-semialdehyde → proline.
glutamic acid → N-acetyl-glutamic acid → N-α-acetylornithine
ornithine → citrulline → arginine

I

diaminopimelic acid ↓ lysine ← aspartic acid → homoserine → threonine ↑ methionine
α-ketobutyric acid → α-keto-β-methylvaleric acid
⇵ ⇵
α-aminobutyric acid isoleucine

II

Organisms which can meet all their carbon requirements from two-carbon compounds such as alcohol and acetate possess a mechanism known as the glyoxylate cycle for achieving a net synthesis of C_4-dicarboxylic acids from acetic acid.[258–260] Thus acetate enters the cycle, reacts with oxalacetate to form citrate; citrate is transformed to cis-aconitate which in turn is changed to isocitrate; succinate leaves the cycle in a reaction in which glyoxylate is also formed; glyoxylate combines with activated acetate at this point to yield malate which in turn is oxidized to oxalacetate thus completing the cycle. One mole of succinate is produced for each two moles of acetate entering the cycle. Intermediates removed from the citric acid cycle can be regenerated in the glyoxylic acid scheme. The modified citric acid cycle occurs in species of *Pseudomonas*, in molds, and in many strains of *E. coli*.

The Pasteur Effect

The aerobic dissimilation of carbohydrates represents a more thorough-going oxidation of substrate than the anaerobic one. The much greater store of energy released becomes available through the

instrumentality of "energy-rich" phosphate bonds for those assimilatory processes of the cell which require energy. This means that for a given amount of substrate, more cells will be produced in aerobic dissimilation. Pasteur was the first to generalize on this behavior, and consequently the action of air in effecting increased cellular development, or conversely, diminished glycolysis, is known as the Pasteur effect. The effect is peculiar to facultative organisms; and if we wish we may utilize the Pasteur effect to define and limit this group, i.e., the facultative microorganisms are those which exhibit a Pasteur effect.

Current views would have the phosphorylation reactions occurring during respiration compete with the coupled dehydrogenation and phosphorylation of phosphoglyceraldehyde.[230,320,323] The supply of inorganic phosphorus required for the phosphorylation of phosphoglyceraldehyde becomes exhausted, and glucose fermentation is inhibited. The lower uptake of glucose during respiration as compared with that during fermentation is attributed to the lack of adenosine triphosphate at the site of glucose phosphorylation. Such adenosine triphosphate as is formed accumulates in the mitochondria, and is therefore not readily available for glucose phosphorylation.

Fat Metabolism

Although fats as a source of carbon and energy are not as important in microbial as in tissue metabolism, the fact remains that microorganisms may contain within their cytoplasm fat bodies which, in mycobacteria and in fungi such as *Geotrichum candidum* (*Oospora lactis*), constitute a weighty proportion of the dry microbial mass. We may presume that fats constitute a reservoir of carbon and energy upon which the highly aerobic organism may draw under starvation conditions. Many of the flavors observed in highly-flavored cheeses, such as Roquefort, Gorgonzola, Camembert, and Limburger, have their origin in the breakdown of lipids.

Fat normally occurs dispersed as globules of microscopic dimensions, and, unless lipolyzed, will remain unavailable to the cell. Many microorganisms have been found to contain lipases.[4,73] Collins and Hammer[73] isolated 159 lipolytic bacterial cultures from various sources—all of which could conceivably contaminate a milk supply. Lipolytic organisms often present in milk include *Achromobacter lipolyticum*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, and *Achromobacter lipidis*.[4,105,168,222] Also, yeast of the genus *Torula* and most molds have been found to possess lipolytic activity.[435]

Lipolytic activity and the important part it plays in flavor and texture development in cheese has been the subject recently of extensive investigations.[58,169,211,212,394,493] Girolami and Knight[138] found that resting cells of *Penicillium roqueforti* oxidized fatty acids in the presence of phosphate and magnesium to respective methyl ketones with one less carbon atom. More recently Jackson and Hussong, studying blue cheese, isolated a number of secondary alcohols—the oxidation products of pentanone-2, heptanone-2, and nonanone-2.[224]

Lipolysis makes available to the organism glycerol and a large variety of fatty acids, saturated and unsaturated, of different chain lengths. Glycerol, it may be presumed, is decomposed according to the Meyerhof-Embden scheme, entering the pathway to pyruvic acid at the point corresponding to triose formation in the decomposition of glucose. It is dehydrogenated, a reversal of the step leading to its formation from phosphoglyceraldehyde, and ultimately its three-carbon "backbone" appears in pyruvic acid. The oxidation of glycerol makes available for subsequent reduction reactions four hydrogen atoms compared with the two made available in the oxidation of trioses. The difference reflects the lower degree of oxidation of glycerol. Generally substrates possessing a relatively low degree of oxidation give rise to end products which in toto represent a relatively low degree of oxidation. The degree of oxidation of n moles of an organic substance with the generalized formula $C_xH_yO_z$ may be represented by the number $n\ (y\text{-}2z)$.[295] In anaerobic decomposition, the number representing the degree of oxidation of the substrate must be equal to the sum of the numbers representing the degree of oxidation of the end products.

Metabolism of Fatty Acids.—A long time elapsed before the observations of Knoop[249] on the basic principles underlying the β-oxidation of fatty acids were extended to show that the CO_2 involved in β-oxidation was formed in the citric acid cycle.[37,281,360,535] This meant by analogy, that an active 2-C compound might play an important role in fatty acid oxidation. This intermediate is acetyl-CoA, and an early indication of its role in fat metabolism was suggested by the studies of Barker,[14] Burton[48] and Lynen[324] in connection with the reversible series of reactions leading from acetyl-CoA to butyric acid in the butyric acid fermentation.

We shall now consider the general mechanism for the oxidation of fatty acids. In the first stage, an acyl-coenzyme A compound is formed. The reaction may require "sparking" by acetyl-coenzyme A, or it may take place in the presence of ATP and coenzyme A. In the presence of acyl dehydrogenase and flavin-adenine-dinucleotide,

dehydrogenation occurs, and saturated acyl-CoA is converted to the corresponding unsaturated acyl-CoA compound with the double bond located between the α and β carbon atoms. Hydration in the presence of enoyl hydratase then yields L-β oxyacyl-CoA, which in turn is oxidized in the presence of β-oxyacyl dehydrogenase and DPN^+ to yield β-ketoacyl-CoA. This compound then undergoes thiolysis in the presence of CoA and β-ketoacylthiolase. Acetyl-CoA and an acyl-CoA compound are formed. The chain length of the new acyl compound is two carbon atoms shorter than the original one. Acetyl-CoA is available for a renewal of the cycle, and in each such renewal acetyl-CoA and an acyl-CoA compound bereft of two carbon atoms are formed. Acetyl-CoA becomes available, as a result of such β-oxidation, for condensation with oxalacetate to yield citrate; thus "active acetate" enters the citric acid cycle, and what emerges are the oxidation products, carbon dioxide and water. Unless oxalacetate is renewed to replace the quantity transformed into precursors of amino acids, "active acetate" will not renewedly enter the citric acid cycle. Normally, oxalacetate is renewed as one result of the dissimilation of carbohydrate. Pyruvate or phosphopyruvate, resulting from the fermentation of carbohydrates, reacts with carbon dioxide, and oxalacetate is formed. If for some reason the link between carbohydrate and fatty acid metabolism is disturbed, two molecules of acetyl-CoA combine in the presence of β-ketothiolase to yield acetoacetyl-CoA, and this compound in turn yields, upon enzymatic hydrolysis, acetoacetic acid.

Biosynthesis of Fats.—Carbohydrates comprise the primary source of material and energy for the biosynthesis of fats. Decomposition of carbohydrates results in the formation of pyruvic acid and two molecules of reduced coenzyme I. The oxidative decarboxylation of pyruvic acid then yields acetyl-CoA, carbon dioxide and a second molecule of reduced coenzyme I. With the formation of acetyl-CoA, the stage is set for the synthesis of higher fatty acids by way of the fatty acid cycle. The sequence of reactions leading to the β-oxidation of fatty acids is reversed. Two molecules of acetyl-CoA condense to yield acetoacetyl-CoA, which in turn accepts hydrogen to form L-β-oxybutyryl-CoA. This oxy-compound then undergoes dehydration followed by reduction to yield butyryl-CoA. Acetyl-CoA enters the cycle for the second time, and reacts with butyryl-CoA. The hydrogenation, dehydration and reduction steps, in the order indicated, are repeated, and an acyl-CoA compound is formed in which the acyl component now contains six carbon atoms. This process, repeated often enough, leads to the formation of a pool

of acyl-CoA compounds with acyl components of varying chain length, each chain of which, however, is unbranched and contains an even number of carbon atoms. The biosynthesis of fatty acids with an odd number of carbon atoms requires a primary condensing unit that contains an odd number of carbon atoms. This primary unit is propionyl-CoA. It is formed in the rumen of ruminants by the action of bacteria on cellulose. Propionyl-CoA is resorbed from the blood and made available for synthesis. As one might expect, the fat of ruminants has been found to contain fatty acids with an odd number of carbon atoms.

In the synthesis of fats, the "energy-rich" acyl-CoA compounds are seldom hydrolyzed. Rather, the energy associated with these compounds is utilized in the synthesis of ester bonds. Esterification of 3-phosphoglycerol with two molecules of acyl-CoA leads to the formation of CoA and phosphatidic acid, a precursor of fats, thus:

$$2\ R\cdot COSCoA + CH_2OH\cdot CHOH\cdot CH_2OPO_3H_2 \rightarrow CH_2OR\cdot CHOR\cdot CH_2OPO_3H_2 + 2\ HSCoA \quad (R68)$$

Kornberg and Pricer[261] studied the enzymes in liver involved in this transacylation, and have made the interesting observation that the relative activity of various acyl-CoA substrates plotted against chain length is maximum for fatty acid derivatives containing 16 to 18 carbon atoms. This would account for the predominance of palmitic, stearic, and oleic acids in the fat of animal tissues.

Coenzyme A in fat metabolism appears to act in a multifunctional manner. Compound formation with fatty acids, renders more reactive the hydrogen atoms in the neighborhood of the carboxyl group. Spontaneous condensation of two molecules of acetic acid to acetoacetic acid under physiological conditions is thermodynamically impossible. Condensation of two molecules of acetyl-CoA to acetoacetyl-CoA is possible. Therefore, both condensation and the reverse reaction can occur on the same enzyme surface only with activated acyl groups, such as acyl-CoA provides. Compounds of the insoluble higher fatty acids and coenzyme A are soluble in water, and thus the acyl groups are more accessible to chemical attack.

Characteristic differences between thioesters and the corresponding oxygen esters have been discussed by Lynen.[321] The much larger nuclear charge on the sulfur atom would increase the positively charged character of the carbon associated with it in thioesters, and as a consequence, it would be readily susceptible to attack by nucleophilic substituents (Lewis bases) and carbanion containing molecules.

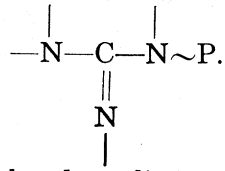
Malonyl-coenzyme A.—A recent and remarkable development stems out of the observation of Klein *et al.*[245,246] that homogenates of anaerobically grown *Saccharomyces cerevisiae* cells incorporated five times as much acetate into fatty acids whenever CO_2 was not removed from the aerobic environment of the experiment. A related observation by Gibson *et al.* specifying a bicarbonate requirement for the synthesis of long chain fatty acid was reported almost simultaneously.[137] Commenting on these observations, Lynen[321] proposed that reaction between bicarbonate and acetyl-CoA would lead to the formation of a malonyl-CoA intermediate, and that it was malonyl-CoA rather than the acetyl-compound which was active in the synthesis of β-keto acids. A decarboxylation would attend the condensation of malonyl-, and acetyl-CoA, and the accompanying energy release, Lynen reasoned, would shift the thiolase equilibrium in favor of the β-keto acid. Moreover the observation that biotin is necessary for the synthesis of palmitic acid, and the likelihood that biotin is involved in all biochemical carboxylation mechanisms supported Lynen in his thesis. This interesting hypothesis was confirmed by Lynen and his collaborators and malonyl-CoA was isolated in experiments with crude enzymes prepared from cell-free yeast homogenates and systems containing unlabeled acetate and radioactive bicarbonate.

Phosphate Bond Energy

The energy available to the microorganism for the performance of its vital functions (assimilation, reproduction, movement, etc.) is derived from the free energy changes attending oxidation of the substrate. Energy is to a large extent not wasted as kinetic energy, but is stored as molecular energy as a result of coupled reactions, bimolecular or unimolecular, leading to the formation of "energy-rich" phosphate bonds. Lipmann,[310] in 1941, elucidated the function of compounds containing these bonds thus: "During various metabolic processes, phosphate is introduced into compounds not merely or at least not solely to facilitate their breakdown, but as a prospective carrier of energy."

Three types of compounds have been observed to contain "energy-rich" phosphate bonds. Pyrophosphates contain such a bond, thus: —P—O~P. The slurred line indicates an "energy-rich" bond. The mixed anhydride type, embracing a carboxy-acid and phosphoric acid, is denoted by $-\overset{\overset{\|}{O}}{C}-O\sim P$, and includes types derived from the fundamental type by substitution of bivalent groups for

carboxyl oxygen. Finally, a type is found in which phosphoric acid is linked with guanidine, or derivatives of guanidine, thus:



—N—C—N∼P. The designation of these bonds as "energy-rich" bonds to distinguish them from bonds of the simple ester type signifies that they are relatively labile with respect to hydrolysis. Inorganic pyrophosphates, for example, dissociate almost completely although slowly into orthophosphates even at room temperature. Thermodynamic considerations lead one to regard the "energy-rich" bond as relatively labile; considerations of reaction kinetics lead one, on the contrary, to associate a relatively high activation energy requirement with the severance of this bond. Lipmann[313] explained that the phosphate bond is so well suited for its biochemical duties because the relatively high activation energy requirement serves to retard rapid hydrolysis and to confer a measure of stability to thermodynamically labile compounds.

Coupling of Oxidation with Phosphorolysis

The formation of "energy-rich" phosphate bonds results from the coupling of an oxidation-reduction reaction with phosphorylation. This reaction may be intramolecular, as for example in the formation of phosphoenolpyruvic acid from 2-phosphoglyceric acid (see R11), or it may be intermolecular involving usually Co I or Co II, as in the phosphorylative oxidation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid (see R7).

If the entire output of energy in aerobic glucose oxidation is converted into phosphate-bond energy, each bond possessing 15 kilocalories, some 45 phosphorylations would result. The ratio between the quantity of inorganic phosphate converted to organic phosphate and the amount of oxygen consumed in oxidative phosphorylations—the P/O ratio—is an important index of the efficiency by which the energy of oxidative processes is converted into available energy. The true P/O ratio was estimated to be 3.0 in the complete oxidation of pyruvate by way of the citric acid cycle.[22,384] It appears that in addition to the phosphorylation of substrate at any level, phosphorylation of intermediates in the stepwise approach to the final oxidant may and does occur. Direct experiments have established that phosphorylation is coupled to the aerobic oxidation of reduced diphosphopyridine nucleotide.[284]

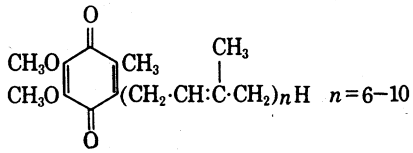
Inorganic ions, and the permeability and structure of mitochondria are important factors in oxidative phosphorylation.[243,278,282,283]

The mechanism for the storage in and transfer of energy through "energy-rich" phosphate bonds is not unique. In the oxidation of pyruvic acid, an anhydride bond rich in energy is formed between an "active" acetyl group and a sulfhydryl group of coenzyme A. Such compounds enter readily into reactions with inorganic phosphate to yield "energy-rich" phosphate bonds; acetylphosphate, for example, forms readily in the reaction between acetyl-coenzyme A and inorganic phosphate.

Recent years have witnessed an increasing number of demonstrations that "energy-rich" compounds are utilized in cellular growth. Such assimilatory reactions as peptide, urea, and glutamine synthesis, and indirectly polysaccharide formation by way of hexoses, have been studied.[28,66,184,466]

Coenzyme Q

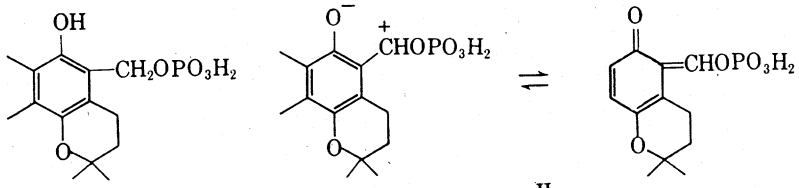
A group of quinones (related to napthoquinones and vitamin K) occur widespread among microorganisms, especially aerobic ones. These act as coenzymes by undergoing cyclic oxidation and reduction during substrate oxidation in mitochondria and are involved in oxidative phosphorylations.[81,293,357] The coenzyme has been characterized as a group of 2,3-dimethoxy-5-methylbenzoquinones, thus:[541]



Members of the coenzyme Q group are designated as $Q_6 \ldots\ldots Q_{10}$ depending on the value of n.

Coenzyme Q is very likely an additional member of the respiratory chain concerned with the oxidation of succinate.[81A,187,188,284A] During phosphorylative oxidation coenzyme Q is concomitantly involved in the energization of inorganic phosphate with the formation of an energy-rich phosphate bond.[187,188] Model compounds such as the quinol phosphates have been found to acquire a phosphorylating function on oxidation.[63] Currently, the structural type of phosphate based on a 1,2-pyran ring skeleton is regarded as more significant than the type related to dihydrocoenzyme Q_{10} monophosphate. Folkers *et al.* considers that the 5-phosphomethyl-6-chromanol type I shown below can be oxidized to system II which then participates

in oxidative phosphorylation by generating metaphosphate (HPO_3).[114,504]



I II

Polysaccharide Formation

The condition known as ropy or slimy milk, the result of an abnormal fermentation, is encountered not infrequently in milk in epidemic proportions. Its immediate cause is the formation of gums with a polysaccharide character, and mucins. The mechanism of polysaccharide formation is discussed herewith; practical aspects are considered later.

Free Energy Changes.—The free energy change accompanying the hydrolysis of sucrose is approximately −6,500 calories per mole; the corresponding free energy change in the hydrolysis of lactose is approximately 2.5 times as great. Hydrolysis is favored, and in order to reverse the reaction under physiological conditions, i.e., in neutral dilute aqueous solution, the synthesis of lactose or sucrose must be coupled with an energy-yielding reaction. Much the same condition prevails in the hydrolysis of such polysaccharides as glycogen and starch.

Synthesis of Polysaccharides.—There are two known pathways in the synthesis of polysaccharides; one requires the mediation of phosphorylase and phosphate, the other does not. The phosphorolytic pathway will be considered first.

Polysaccharide phosphorylases have been found in animal tissues, yeast and bacteria.[55,185,194] These enzymes may catalyze either the scission or the formation of α-1,4-glucosidic bonds at the nonreducing end of a glycogen or starch chain. In synthesis, these enzymes foster a metathetical reaction between glucose-1-phosphate and the polysaccharide chain. Scission occurs between the carbon and oxygen of the phosphorylated hexose group, the hexose moiety is transferred to the nonreducing end of the polysaccharide chain, and a proton from the chain end is transferred to the phosphate moiety. No net change in free energy is involved, inasmuch as the free energy changes attending the hydrolysis of both glucose-1-phosphate and starch or glycogen are approximately equal. As the H-ion concentration of an

equilibrated solution decreases, the concentration of the stronger acid, glucose-1-phosphate, increases. The reaction requires the presence of a "primer" (a small quantity of starch, glycogen, or dextrin on which chain expansion can take place). The chain prolongation eventually ceases, and the addendum separates from its "primer."[78]

The Transglycosidases.—The transglycosidases are enzymes which promote the transfer of hexose units from di- to monosaccharides, from di- to oligosaccharides, and from di- to polysaccharides.[193] In these reactions, the free energy of hydrolysis of disaccharides is utilized in an interesting manner to effect the synthesis of oligo- and polysaccharides. Lactase possesses an interesting transgalactosidylase function which will be described in the section on lactose metabolism.

The Glucose Donor-Uridine Diphosphate Glucose.—In a long series of recent papers, Leloir and his collaborators have described a number of reactions in which uridine diphosphate-glucose (UDP-glucose) acts as a glucose donor to produce the following: trehalose, sucrose, sucrose phosphate, bacterial cellulose, and glycogen. There is evidence according to these investigators that in animal tissues, glycogen is synthesized from UDP-glucose and not from glucose-1-phosphate.[288–291]

The occurrence and postulated occurrence of UDP-glucose in all lactose fermenting microorganisms suggests that this compound may be involved in microbial polysaccharide synthesis.

Assimilation—Material and Energy Balance

Assimilatory processes among which polysaccharide synthesis is an example are endoergonic processes whereby the cell reproduces the macromolecular species and essential metabolites required for its renewal and survival. These processes are commercially significant in the microbiological production of the cells themselves as in yeast manufacture and in the production of cellular constituents such as fats, polysaccharides, and vitamins. They are important in sewage disposal where a balance must be maintained between cellular synthesis and cellular losses (endogenous respiration).

General Considerations.—The efficiency of an assimilatory process is usually described in terms of a number of coefficients—the conversion, the fat, the protein, and the carbohydrate coefficient. These denote in turn the weight of cellular dry substance, the weight of fat, the weight of protein, and weight of carbohydrates (essentially polysaccharides) which are obtained per 100

gm. of an active source of carbon present in the medium. For comparison purposes, if glucose, for example, is the source of carbon, the determination is best made at the time of complete glucose utilization.

The consensus at present considers the active two carbon compound, acetyl-CoA as the building block for the preponderance of fat and protein found in nature, although there are some, notably Clifton, who regarded a one carbon fragment, an "active" formaldehyde as the precursor.[64] Both schemes would make available for assimilation two-thirds of the total glucose present; the remaining third would be used in fermentation.[118]

The Maximal Values of the Conversion Coefficient.—Dry yeast on an average contains between 45–47% carbon.[482] One hundred grams of glucose contains 67 gm. assimilable sugar, the carbon content of which is approximately 27 gm. The maximum quantity of dried yeast containing 45–47% carbon which can be realized from 100 gm. glucose (the maximum conversion coefficient) lies therefore, between 57–60. Roughly, about 50% of the dry cellular material produced under normal conditions of culture is protein; hence the maximum value of the protein coefficient lies in the neighborhood of 30.

The Maximum Value of the Fat Coefficient.—If the energy available for synthesis is assumed constant, it is possible to draw conclusions concerning the maximum value of the fat coefficient.[427,456] Assuming that the microorganism is yeast and that it is composed of fat, protein, ash, and carbohydrates with respective heats of combustion relative to glucose of 2.5, 1.7, 0, and 1 and with respective percentages of fat, protein, ash, and carbohydrate in the dry yeast of F, P, A, and 100—F—P—A, and assuming that the conversion coefficient is E, one may conclude in virtue of the assumed constancy of the available energy that the following relationship would hold, thus: $2.5\ F + 1.75\ P + 100—F—P—A \leqq (77 \times 100/E)$. It has been found that the heat of combustion of normal yeast containing about 50% protein and 5% fat is approximately 77% that of the assimilable sugar; hence 77 occurs in the right-hand member of the equation. The equation shows that if the fat content of the yeast is increased at the expense of either the protein or carbohydrate, the maximum value of the conversion coefficient i.e., the yield of dry cells per 100 gm. of glucose must decrease. Moreover as the fat content of the cells increases, so must the maximum value of the fat coefficient.

Theoretically if all of the assimilable sugar is converted into glycerol, water, and fat with the tripalmitin composition the resulting

cellular mass would contain 29.8 gm. fat for each 100 gm. of processed sugar.[116] Since, however, an appreciable part of each cell consists of protein and other nonfat constituents, fat coefficients greater than 15 are not observed in practice.[158]

FERMENTATIONS IN MILK

Fermentations in milk modify its properties favorably or adversely, giving rise to such useful products as starter culture, cultured beverages and cheese on the one hand, and to spoiled and degraded products on the other. Some fermentations lead to the development of antibiotics which may, depending on the circumstances, be desirable or undesirable. Fermented milk products are unique in the sense that the acquired organoleptic properties (reflecting as it does a distinctive blending of flavor-imparting components and distinctive body qualities) depend on the unique characteristics of certain constituents of milk, the curdling properties of the calcium caseinate-phosphate complex, the blandness of lactose, and the flavor characteristics of lipolysed milkfat, for example. This section is devoted to the following topics: milk (raw and heated) as a fermentation medium, lactose and its dissimilation, citrate and its dissimilation, nitrogenous compounds in milk, proteolysis, cultured milks, and undesirable fermentations in milk.

Milk as a Fermentation Medium

Milk is a versatile fermentation medium. It contains the elements and substrates required even by such nutritionally exacting organisms as the lactobacilli. It is not a universal medium, however. It contains bacteriostatic substances among which are the mild easily-destroyed lactenins of historic interest.[231,232] The disaccharide lactose is the chief source of carbon, and thus the application of milk is limited to fermentations for which lactose-fermenting organisms are available; or at best additional and uneconomic processing becomes necessary for the conversion of lactose to fermentable monosaccharides. In the propionic acid fermentation, supplementing of milk or whey with a source of degraded protein is necessary in order that maximal fermentation rates may be achieved. Milk normally is deficient in manganese with respect to the requirements of lactobacilli for maximal growth rates. It normally is also deficient in iron with respect to the requirements of certain species of *Clostridium* for the production of volatile solvents and riboflavin. It contains only trace quantities of cobalt, which do not suffice for maximal microbial synthesis of vitamin B_{12}.

On the other hand, milk is a rich source of vitamins and organic growth factors. It is especially rich in riboflavin and orotic acid. Supplementation of lacteal media with vitamins or extracts is only rarely necessary. The proteins of milk contain in superabundance all of the amino acids essential for bacterial growth. Proteoses and amino acids are present in sufficient quantities to promote the onset of most fermentations.

The heat treatment milk undergoes prior to most fermentations changes its characteristics as a culture medium. Milk autoclaved 15 min. but not longer was reported superior to milk heated at 80°C. for 10 min. for culturing lactic streptococci.[122] Heating milk at 62°–72°C. for 30 to 40 min. was reported stimulatory to growth of starter cultures.[147–149] Heating at 72° for 45 min., at 82° for 10–45 min., or at 90°C. for 1–45 min. inhibited growth. Drastic heating beyond the limits mentioned stimulated growth. Believed to be responsible for the initial stimulation were the following: oxygen expulsion, destruction of inhibitors, partial protein hydrolysis, and serum protein denaturation. Inhibition was associated with the formation of toxic volatile sulfides, and the succeeding stimulation with the heat-induced disappearance of these sulfides.[147–149]

Lactose, fat, and citric acid comprise the important fermentable (C, H, O) compounds of milk. Fat metabolism has been considered in a general way and will not be discussed further. Citric acid metabolism has been considered only indirectly in connection with the citric acid cycle. Lactose metabolism follows essentially the same course as glucose metabolism. There are, however, distinct features involved in its transformation to the required intermediate compound, glucose-1-phosphate.

Lactose and Lactase.—Lactose, milk's chief source of energy for microbial metabolism, is a dissaccharide constituting about 40% of the solids of whole milk. Strictly speaking, lactose is α-d-glucopyranose-1, 4-β-d-galactopyranose.

The preponderance of evidence shows that in bacterial metabolism lactose is first hydrolyzed to glucose and galactose. The enzyme which brings this about, lactase or more properly, β-galactosidase, has been found in lactose-fermenting yeasts, molds, *E. coli*, *A. cloacae*, and the intestinal wall of the calf.[228] The older literature refers to lactase as an endoenzyme in "true lactics"[388] and to the presence of lactase in *L. bulgaricus*,[35] *S. lactis* var. *taette*[156] and *E. coli*,[505] among others.

Lactase functions both as β-galactosidase and as a transgalactosidylase; that is, lactase not only brings about hydrolytic cleavage of

lactose, but also transfers galactosidyl residues to various acceptors, including water, glucose, galactose, lactose, and oligosaccharides. In solutions containing high concentrations of lactose, the enzymatic reactions may even favor the formation of oligosaccharides at the expense of the monosaccharides, glucose and galactose.[8,391,392,431,432,507,508]

One enzyme, and not two, is involved in the formation of monoses and oligosaccharides.[391,392] Using a commercial preparation from lactose-fermenting yeasts, Pazur studied the products and postulated the following sequence of reactions:

$$\text{glucose} \overset{1,4}{—} \text{galactose} + \text{lactase} \rightarrow \text{galactose-lactase} + \text{glucose} \quad \text{(R69)}$$

$$\text{galactose-lactase} + \text{glucose} \rightarrow \text{glucose} \overset{1,6}{—} \text{galactose} + \text{lactase} \quad \text{(R70a)}$$

$$\text{galactose-lactase} + \text{galactose} \rightarrow \text{galactose} \overset{1,6}{—} \text{galactose} + \text{lactase} \quad \text{(R70b)}$$

$$\text{galactose-lactase} + \text{glucose} \overset{1,4}{—} \text{galactose} \rightarrow \text{glucose} \overset{1,4}{—} \text{galactose} \overset{1,6}{—} \text{galactose} + \text{lactase} \quad \text{(R70c)}$$

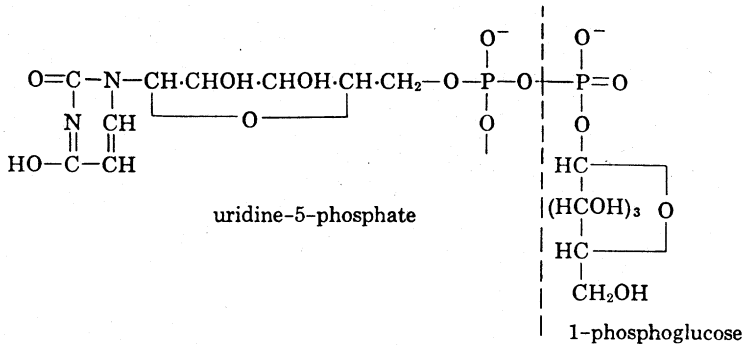
$$\text{galactose-lactase} + \text{glucose} \overset{1,6}{—} \text{galactose} \rightarrow \text{glucose} \overset{1,6}{—} \text{galactose} \overset{1,6}{—} \text{galactose} + \text{lactase} \quad \text{(R70d)}$$

$$\text{galactose-lactase} \xrightarrow{+H_2O} \text{galactose} + \text{lactase} \quad \text{(R70e)}$$

This mechanism was supported by the results of experiments with the following: labeled lactose, lactose plus labeled glucose, and lactose plus labeled galactose. In agreement with Pazur's conclusions were those of Wallenfels *et al.*,[507,508] who worked with preparations from molds, *E. coli* and calves' intestines.

Roberts *et al.*,[431,432] found at least eleven oligosaccharides among the products of the reaction; with concentrated lactose solutions, high yields of oligosaccharides were obtained.

The Conversion of 1-Phosphogalactose to 6-Phosphoglucose.—Following a suggestion of Kosterlitz,[263,264] Caputto *et al.*[54] noted that the conversion was accelerated by two factors, one thermolabile, the other stable. The thermostable factor present in purified yeast extract was uridinediphosphate-glucose; the thermolabile factor was identical with purified phosphoglucomutase, the enzyme catalyzing the conversion of 1-, to 6-phosphoglucose. Uridine-diphosphate-glucose (UDPG) has been isolated and has been assigned the following formula:[54]



The structure was confirmed by the splitting-off of glucose and one norganic phosphate group on milk acid hydrolysis.

Twenty-five per cent of UDPG in a crude enzyme preparation is onverted on incubation to uridine-diphosphate-galactose. Based n this observation, the following scheme was proposed as a working ypothesis.[287]

$$\text{galactose} + \text{ATP} \rightleftharpoons \text{galactose-1-phosphate} + \text{ADP} \qquad (\text{R71a})$$

$$\begin{array}{c}\text{galactose-1-phosphate} + \text{UDP-glucose} \\ \downarrow\uparrow \\ \text{glucose-1-phosphate} + \text{UDP-galactose}\end{array} \qquad (\text{R71b})$$

$$\text{UDP-galactose} \rightleftharpoons \text{UDP-glucose} \qquad (\text{R71c})$$

Reaction 71a is catalyzed by a galactokinase present in galactose-dapted yeasts and in *L. bulgaricus*. The occurrence of step b is at resent largely a matter of conjecture. Step c is catalyzed by an nzyme named galactowaldenase. The reaction is essentially a Valden inversion, at C-4, of glucose. Concerning its mechanism eloir[287] concluded "It is impossible at present to correlate the knowl-dge provided by organic chemistry with the enzymatic reac-ions . . ."

Hansen and Craine[174] and Rutter and Hansen[444] separated the nzyme system containing galactowaldenase from that containing hosphoglucomutase. The waldenase was active only with respect o α-galactose and α-glucose. The β-sugars were not attacked. he equilibrium between UDP-glucose and UDP-galactose was such hat 73–79% of the equilibrium mixture consisted of UDP-glucose.

Adaptation of Organisms to Lactose.—Many microorganisms ontain enzymes necessary to bring about the transformation of actose to 6-phosphoglucose and they are said to possess the enzymes onstitutively. Other microorganisms can be adapted to grow on actose on the basis of models furnished by the presence of lactose,

galactose, or lactose-like and galactose-like compounds; and such organisms in which the ability to ferment lactose is acquired are said to possess the enzymes adaptively.[70] The model substances, the presence of which is required for induced enzyme synthesis, are called inductors. Inductors have the interesting property that if present in a medium they will induce the synthesis of all of the enzymes involved in their decomposition, whether some participating enzymes are present constitutively or not.

The elucidation of the mechanism of induced synthesis is receiving much consideration.[70] A source of energy and a source of amino acids are required. Sometimes the energy can be supplied only under oxidative conditions, as in the synthesis of the galactozymase system in yeast. Adaptive enzymes are not ususally perpetuated in serial transfers unless the essential substrate is always present. Organisms trained to ferment lactose over a long series of transfers appear to lose the ability after a few transfers in media devoid of this substrate.

Direct Versus Indirect Lactose Utilization.—Studies on relative fermentation rates of disaccharides and component monosaccharides have always lent themselves to interpretation on the basis of selective permeation effects. An alternate view considers the differences in rates as evidence that disaccharides can be dissimilated directly.

Lactose is fermented by various yeasts at a uniform and more rapid rate than equimolar mixtures of glucose and galactose.[285,362,437,524] Moreover, Myrbäck *et al.* observed that if toluene was used to suppress fermentation, hydrolysis of lactose in the presence of toluene proceeded more rapidly than fermentation in its absence.[362] In order to account for differences in fermentation rates, it seemed reasonable to postulate that lactose could be dissimilated directly. Moreover, in acid solution hydrolysis was suppressed but fermentation was not. Myrbäck argued that lactase was localized in the cell wall and hence was subject to the influence of the pH of the medium, whereas the organism's apparatus for direct dissimilation was located in the interior where the pH might conceivably differ locally from that of the medium.

The theory of direct dissimilation disregards the occurrence of specific permeability effects and the effect of toluene on permeability.[135A] The consensus at present considers the lactase function to be indispensable.

Recent concerted attacks on problems pertaining to the selective permeation of the cell favors the idea of stereospecific permeation sys-

tems which determine whether neutral molecules will have access into the cell and at what rate.[65,425,465] Suspensions of *E. coli* containing the inducible enzyme β-galactosidase will hydrolyze lactose but not thiogalactosides (lactose analogues in which the oxygen atom of the glycosidic linkage is substituted by sulfur) and thus the thiogalactoside can accumulate within the cell to an intracellular concentration of galactoside which may exceed by 100-fold or more its concentration in the external medium. To account stoichiometrically and kinetically for the rates and amounts of accumulation, a stoichiometric and a catalytic model were compared. Only the catalytic or permease model, which assigned to the specific permease sites, the role of catalyzing the accumulation of the galactosides within the cell rather than serving as the final acceptors, proved capable of accounting successfully for the observed reaction kinetics. To account quantitatively for the accumulation of galactoside in the cell, it was necessary to take into consideration the evidence that cells genetically or otherwise devoid of permease were incapable of metabolizing lactose although they possessed β-galactosidase activity. Thus the correct model appeared to be one in which the permeation barrier in the absence of permease possessed a high degree of impermeability toward carbohydrates, and the catalytic permeases if present would be associated with the barrier (not necessarily the cell wall). Thus the rate of permeation into the cell would depend on the external concentration, on the leakage rate and above all on the activity of the permease. The rate of exit from the cell would depend on the concentration of free substrate within the barrier and on the leakage rate.

Citric Acid

The importance of citric acid is out of all proportion to the small quantity (0.07 to 0.4%) present in milk. Citric acid is an essential substrate for the desirable aroma-producing organisms of milk products. Its decomposition gives rise to the flavors characteristic of butter. Bosworth and Prucha[30] observed that the citric acid in milk disappeared during the normal souring process. Kickinger[242] showed that the citrates in milk disappeared as a result of the action of bacteria which survived heating to the boiling point.

Hastings *et al.*[186] observed that the breakdown of citrates in milk and in synthetic media did not follow parallel courses. The difference brought about by the use of synthetic media was marked if citrates were the sole source of carbon. According to these workers, the nature of the available carbon determines the course of citrate metabolism. Citric acid is fermented by a large number of organ-

isms which may find their way into milk, for example, by *E. coli*, *A. cloacae*, *A. aerogenes*, *L. citrovorum*, *L. dextranicum*, *B. subtilis*, *Proteus vulgaris*, *L. casei*, *L. acidophilus*, *L. bulgaricus*, alkali-forming bacteria, and lactose-fermenting yeasts. Citrate metabolism is discussed under the heading "Fermentation by Starters."

Fermentation by Starters

Lactic acid bacteria are used widely as starters in the cheese industry in order primarily to promote and control acid formation and to initiate the desired fermentation. These bacteria are also useful in butter manufacture, creating acid conditions which inhibit the growth of undesirable organisms and contributing to the production of the desirable flavor and aroma. They also serve much the same purpose in the manufacture of cultured buttermilk, sour cream, and margarine. The predominant organisms present in the usual lactic starter are *S. lactis* and/or *S. cremoris*. Certain aroma-producing bacteria, *L. citrovorum* and *L. dextranicum*, may be present in smaller numbers. *S. cremoris*, *Streptococcus diacetilactis*, and occasionally *Pediococcus* species are relied upon in middle European countries for aroma development.[235]

Formation of Lactic Acid by Starters.—*S. lactis* and *S. cremoris* are the acid-producing species in starters. These organisms are facultative anaerobes; they grow at temperatures as low as 10°C. but not at 45°C. They are homofermentative, and produce large amounts of dextrolactic acid and much smaller amounts of acetic acid. Their growth requirements are comparatively complex. Niven[378] studied 21 strains of *S. lactis*. No less than 14 amino acids were required for prompt growth. Although *S. lactis* can attack casein, it seems to thrive better on simpler compounds, and in milk this preference is met by a small but sufficient supply of proteoses and peptones and, to a lesser degree, by a wide selection of amino acids. Growth and acid production in milk by many lactic acid bacteria may be inhibited by developed rancidity and by the presence of antibiotics, sanitizers, other chemicals, and bacteriophage. The subject is discussed in many reviews.[1A,8A,327A,327B,475A]

If spontaneously soured milk is allowed to stand, especially at a comparatively high temperature, it undergoes a second lactic fermentation induced by nonspore-forming bacilli of the *L. bulgaricus* type. Many of these organisms are quite tolerant to lactic acid, and are known to produce 3.25% acid when grown for one month at 29°C. The pH as a consequence may drop to 3.

In the ripening of a lactic culture, lactic acid is produced to the extent of 0.75 to 1.0%, causing the pH to fall to 4.3–4.7. This acid is chiefly *d*-lactic acid but, because of the activity of *Leuconostoc citrovorum* and particularly of *L. dextranicum*, some *i*-lactic acid is also produced from lactose. A small quantity of acetic acid is produced by *S. lactis* from lactose and a much larger proportion is produced by the *Leuconostoc* species from citric acid.

The Production of Biacetyl.—The production of biacetyl in starter cultures is an associative one. Neither *S. lactis* nor *L. citrovorum* (unless pH is artificially reduced) in the absence of the other, will produce efficiently either biacetyl or AMC.[247] Biacetyl is usually accompanied by larger amounts of AMC. Neither is a stable compound in culture, and they tend to be reduced to the more stable 2,3-butylene glycol (2,3-butanediol) in a reaction favored by optimum conditions for the activity of the culture. At 21.1°C. and in neutralized cultures, both biacetyl and AMC tend to disappear quite rapidly.[167] Maximum production with poor yields of biacetyl and AMC occurs in the pH range 4.1–4.4 in pure cultures acidified with sulfuric acid. Maximum yields in cultures acidified with citric acid are much higher, and are obtained in the pH range 3.7–3.9.[348]

Cox[79Ā] found that the aroma-producing organisms of milk first produced biacetyl and then destroyed it. He also found that although a relatively low pH value in the range of 4.4–5.5 retarded growth, it promoted biacetyl formation. Michaelian *et al.*[348] found that the yield of biacetyl could be increased if oxygen was bubbled through acidified milk containing a pure culture. The addition of pure AMC to the medium effected no further increase in yield, and hence the observed increase was not a result of nonenzymatic oxidation.

Prill and Hammer[410] found the following conditions to be conducive to the production in starter culture of biacetyl in good yields: addition of citric acid, shaking and aeration, holding at low temperatures, and lowering the pH of a ripened culture with sulfuric acid. Brewer *et al.*[38] found that agitation of pure cultures in air at pressures of 30 p.s.i. resulted in increases in yield as high as several hundred per cent.

In buttermaking, the practice of cooling ripened cultures and holding them for 24 hr. in the presence of small quantities of citric acid is recommended if such cultures are intended for inoculation of cooled cream and if further growth is not desired.

In keeping with what has been said in the sections on the butylene glycol fermentation, the citric acid cycle, and experiments with labeled acetate, it seems reasonable to assume that the first steps in

the breakdown of citric acid to biacetyl would be its conversion to acetyl-coenzyme A, pyruvic acid and CO_2, thus:

$$\text{citric acid} \overset{\text{condensing enzyme, CoA}}{\rightleftharpoons} \text{acetyl CoA} + \text{oxalacetic acid} \qquad \text{(R95)}$$

$$\underset{\text{oxalacetic acid}}{\text{COOH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}} \overset{\text{malic enzyme}}{\rightleftharpoons} \text{CO}_2 + \underset{\text{pyruvic acid}}{\text{CH}_3\cdot\text{CO}\cdot\text{COOH}} \qquad \text{(R96)}$$

Inviting as this assumption is, it ignores the observation that with lactic acid bacteria, it is not acetyl-coenzyme A but rather acetic acid which accompanies the formation of oxalacetic acid. Moreover, in general, members of the citric acid cycle are not fermented by starter organisms.[356] Another enzyme is evidently involved, one named citritase which has been obtained repeatedly from bacteria.[183,235] Once pyruvic acid is formed, it can follow one or another of three pathways: it can undergo in alkaline media a hydroclastic cleavage to acetic and formic acids; it can undergo in neutral media a dismutation and a decarboxylation to lactic, acetic, and carbonic acids; or finally it can be the starting point of a series of reactions in neutral and acid media leading to the formation of biacetyl among other products, thus:

$$2\text{CH}_3\cdot\text{CO}\cdot\text{COOH} \rightleftharpoons \text{CO}_2 + \underset{\text{CH}_3\cdot\text{CO}}{\overset{\text{H}}{\overset{\text{O}}{\overset{|}{\text{CH}_3\cdot\text{C}\cdot\text{COOH}}}}} \qquad \text{(R97)}$$

α-acetolactic acid

$$\underset{\text{CH}_3\cdot\text{CO}}{\overset{\text{H}}{\overset{\text{O}}{\overset{|}{\text{CH}_3\cdot\text{C}\cdot\text{COOH}}}}} \rightleftharpoons \text{CO}_2 + \underset{\text{AMC}}{\text{CH}_3\cdot\text{CHOH}\cdot\text{CO}\cdot\text{CH}_3} \qquad \text{(R98)}$$

$$\text{CH}_3\cdot\text{CHOH}\cdot\text{CO}\cdot\text{CH}_3 \overset{-2\text{H}}{\rightleftharpoons} \underset{\text{biacetyl}}{\text{CH}_3\cdot\text{CO}\cdot\text{CO}\cdot\text{CH}_3} \qquad \text{(R99a)}$$

$$\text{CH}_3\cdot\text{CO}\cdot\text{CO}\cdot\text{CH}_3 \overset{+2\text{H}}{\rightleftharpoons} \underset{\text{2,3-butylene glycol}}{\text{CH}_3\cdot\text{CHOH}\cdot\text{CHOH}\cdot\text{CH}_3} \qquad \text{(R99b)}$$

The decarboxylation and condensation to carbonic-, and acetolactic acid would be mediated by two distinct enzymes with cocarboxylase as a coenzyme and Mn^{++} as a catalyst. The dehydrogenation to biacetyl would proceed by way of a flavoprotein and a Fe^{++}-protein mediator with oxygen as the hydrogen acceptor. Acetic acid would arise as an additional product by the oxidative decarboxylation of

pyruvic acid in the presence of carboxylase and pyruvic dehydrogenase.

That the first stage in the decomposition of citric acid is an enzyme-mediated reversal of the condensation reaction involving oxalacetate, and the condensing enzyme, is supported by experiments of Takahashi[481] with *Bacterium succinicum*. Each mole of citric acid was found to yield 2 moles succinic, 1 mole acetic and 2 moles carbonic acid. $C^{14}O_2$ was incorporated into the carboxyl group of succinic acid. The reagent α,α'-dipyridyl inhibited the carboxylation reaction and also the decarboxylation of oxalacetic acid; and thus the evidence suggested that oxalacetic acid was an intermediate in the dissimilation of citric acid. Extracts of ground cells actually brought about the condensation of acetate and oxalacetate to citrate.

There is growing evidence that an "active aldehyde" and α-acetolactic acid are intermediate in acetoin and biacetyl synthesis by starter cultures and that an exogenous source of pyruvate is necessary.[461] Thus to the earlier work of Slade and Werkman with cells of *A. cloacae* (*Aerobacter indologenes*) the work of Juni[234] with cell-free preparation from *A. aerogenes*, and other bacteria, and the studies of Dolin and Gunsalus[100] with *S. faecalis*, the investigations of DeMan,[92] and Mizuno and Jezeski,[356] Anderson,[6] and Busse and Kandler,[50] have been added.

DeMan observed that *L. citrovorum* produced biacetyl in a medium containing citrate and pyruvate but not in a one containing glucose. A reducing compound which reduced ammonium molybdate, and which yielded acetoin on distillation was presumed to be α-acetolactic acid.

Mizuno and Jezeski observed that a mixed strain starter culture in skimmilk and in dialyzed milk fortified with various carbon compounds, produced acetoin in increased amounts, when citric-, pyruvic-, or oxalacetic acids was present. Alpha-ketobutyric acid as expected stimulated the production of the acetoin related compound 3-hydroxy-2-pentanone. Addition of glucose alone yielded negative results; addition of citric acid yielded large quantities of acetoin at pH 5.0 but not at pH 7.0; addition of glucose and citrate together brought about the pH independent synthesis of large quantities of acetoin. Alpha-acetolactic decarboxylase activity was demonstrated in ground cells of a *Leuconostoc* isolate.

Experiments by Busse and Kandler[50] with washed suspensions of *L. citrovorum* (*ATCC 8082*) showed that acetoin was not formed from glucose but was formed in large quantities from pyruvate during fermentation in phosphate buffer. In a simultaneous glucose,

pyruvate fermentation acetoin synthesis was inhibited until the glucose had been largely consumed, and CO_2 production had decreased. The results resemble those of Rowatt[440A] on AMC formation by suspensions of *L. plantarum* acting on pyruvate. The initial rate of CO_2 formation at and below pH 4.0 fell off with time, and catalytic amounts of glucose not only prevented the decrease from occurring but also accelerated tremendously the rate of CO_2 production. The added glucose was converted largely to lactic acid. Similar results have been obtained with *L. arabinosus* by Nossal[380] and by Hunt and Nossal.[220]

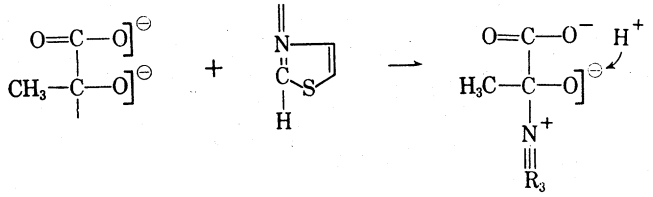
In the experiments of Busse and Kandler, the fermentation of mixed substrates containing glucose-6-C^{14} yielded acetoin, the specific activity of which was only one-eighth of the specific activity of the glucose. Thus pyruvate was the precursor of almost all of the acetoin which was obtained. Busse and Kandler believe that during glucose oxidation sufficient reduced diphosphopyridine nucleotide is available to maintain the concentration of pyruvate (including added pyruvate) at a low level by facilitating its reduction to lactic acid and ultimately to acetic-, and carbonic acids. Thus the decarboxylation of pyruvate with the formation of "active aldehyde" and subsequently of AMC does not occur presumably because of the greater affinity of lactic acid dehydrogenase compared with decarboxylase for pyruvate.

Transformation of Biacetyl.—The fermentation of citric acid does not terminate with the formation of biacetyl and acetoin. Under anaerobic conditions both of the aroma producing compounds are reduced to 2,3-butanediol via appropriate dehydrogenases. Oxygen it appears is capable of competing with both biacetyl and acetoin as a hydrogen acceptor; hence in the presence of oxygen, the aroma producing compounds are conserved. These compounds can also be transformed to acetic acid. Juni and Heym represent the transformation thus: one mole of biacetyl under the influence of thiamine pyrophosphate decomposes into one mole of acetic acid and one mole of "active aldehyde." The "aldehyde" in turn condenses with biacetyl to form diacetylmethylcarbinol. The latter is reduced to acetylbutanediol which in turn decomposes into acetic acid and 2,3-butanediol. Subsequently 2,3-butanediol is oxidized to biacetyl; thus a cycle of reaction is established resulting in the splitting of one molecule of biacetyl into two molecules of acetic acid, and the oxidation of one mole of reduced DPN. Dolin's results with *S. faecalis* indicate that the cycle of transformations would apply to other streptococci.[98]

Some organisms appear to possess a latent acetoin forming capacity. *L. brevis* decomposes pyruvic acid aerobically into acetic-, and carbonic acids, yet in the presence of the inhibitors arsenite (0.01 m) or fluoropyruvate (0.001 m), acetoin and CO_2 are the products.[506]

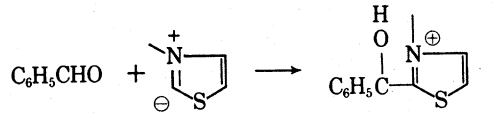
Production of acetoin from citrate requires the combined action of citrate permease and citritase. Thus failure of five strains of *S. diacetilactis* to produce acetoin was linked with their inability to produce citrate permease.[72]

The Nonenzymatic Acyloin Condensation.—Efforts to elucidate the character of the "active" aldehyde precursor of acetoin have persisted for many years. Thus the more general acyloin condensation catalyzed by thiazole compounds including thiamine, was observed by Ugai *et al.* (see reference 49) and later by Mizuhara.[352] The reproduced biological reaction proceeded only if two equivalents of alkali were added per mole of thiamine hydrochloride. A mechanism proposed by Mizuhara and his associates,[353–355] would have the tertiary thiazole nitrogen of the thiamine pseudobase coordinate with the carbo-cation of pyruvate, thus:

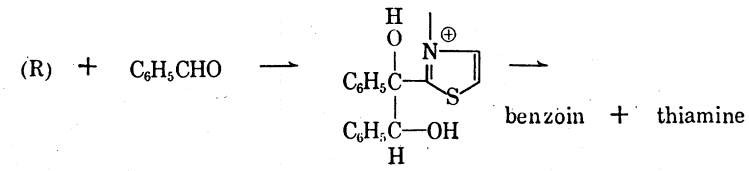


The resulting intermediate would decarboxylate to leave an "active" aldehyde intermediate capable of condensing with acetaldehyde to form acetoin.

Breslow[36] rejected the mechanism based on the reactivity of the tertiary thiazole nitrogen, and proposed instead a scheme in which pyruvic acid first condenses on the N-methylene group of thiamine. This proposal was dropped when experiments with labeled compounds showed that deuterium associated with the methylene group was not lost during condensation. It was subsequently postulated that thiazolium salts are in equilibrium with anions at the C-2 position, and that as a consequence, condensations analogous to the classic cyanide-catalyzed benzoin condensation can occur, thus:



benzaldehyde | thiazole grouping | active aldehyde-thiazolium complex (R)



If acetaldehyde is employed instead of benzaldehyde, an "active" acetaldehyde would be obtained as an intermediate compound.

Krampitz *et al.*[266] recently prepared a compound of thiamine with an α-hydroxyethyl group in the C-2 position and tentatively identified it as an "active" aldehyde.

Nitrogenous Compounds in Milk

The chief nitrogenous compounds in milk are proteins among which one finds two groupings—the caseins and the whey proteins. The caseins are a complex of denatured proteins with a high degree of affinity for each other. In milk they are associated into a complex called the calcium caseinate-phosphate complex which exists as discrete spherical particles ranging in diameter from 10 to 300 mμ. The casein system of proteins coagulates at a pH of approximately 4.6 under the influence of acid, and at the pH of milk in the presence of suitable proteinases, notably rennin and pepsin. The whey system of proteins (about 20% of the total) comprise chiefly β-lactoglobulin, α-lactalbumin, and proteins of the immunoglobulin fraction. β-lactoglobulin, the most abundant of the whey proteins, is denatured when milk is heated to temperatures above 70°C. The denatured protein tends to associate itself with other whey proteins and with members of the calcium caseinate-phosphate complex. Following the coagulation of the caseins with acid, and the whey proteins with heat there remains in the resulting serum a heat-stable proteose-peptone fraction, a variety of amino acids (in some measure reflecting in their relative amounts the amino acid composition of the proteins in milk) and minute amounts of nonprotein nitrogenous compounds such as urea, ammonia, creatine, creatinine, uric acids, etc.

All the free amino acids needed for the effective growth of *S. lactis* are present in milk except phenylalanine and possibly cystine. Glutamine which is stimulatory has not been observed. Variation in milk of a nonprotein nitrogen component (peptide fraction) brings about variations in the growth rates of lactic streptococci.[467]

Proteolysis

The metabolism of amino acids has been briefly discussed in the section on the Krebs cycle. Some organisms can use ammonia as the

sole source of nitrogen, reversing the process by means of which amino acids are broken down to oxoacids. Many organisms active in milk have the apparatus for hydrolyzing the proteins to simpler proteins, polypeptide acids, and amino acids. Gorini studied the elaboration of rennin-like substances (protein coagulating enzymes) especially by *S. marcescens.*[140,141] Bacteria elaborating rennin-like enzymes have the power to "sweet" curdle the casein complex in milk. Thus Hammer and Hussong[173] identified *Bacillus cereus* as the organism responsible for an outbreak of "sweet" curdling in sterile evaporated milk. The coagulum appeared normal to the taste; there was no acid development and no "wheying-off."

Proteinases and peptidases constitute the primary enzyme forms in bacteria responsible for the hydrolysis (proteolysis of milk proteins). Proteolysis is important in cheese manufacture and less so in fermentations in milk. In cheese, proteolysis manifests itself during the long ripening period and is brought about by rennet, the proteinases of milk and probably those of bacteria and molds. Although proteolysis in cheese will be briefly discussed, it is covered more adequately in Chapter 12. Proteolysis is of some significance in the preparation of cultured milks. It obviously plays an important part in the treatment of dairy wastes by aerobic microorganisms. In connection with outbreaks of a bitter flavor defect in evaporated milk, Spitzer and Epple[468] found *Bacillus panis*, a spore-bearing rod with intense proteolytic activity, to be the causative organism.

The role proteolysis plays in cheesemaking may properly be discussed briefly at this point. Proteolysis as carried out by streptococci, and by certain lactobacilli and micrococci is believed to be important in the ripening of Cheddar and perhaps of other cheese types; that carried out by *Brevibacterium linens* is perhaps important in the ripening of Brick, Limburger, and similar cheese, and that brought about by molds is perhaps significant in the ripening of Blue, Roquefort, and Camembert cheese.

Proteolysis by S. lactis.—In cultures of *S. lactis* acid development and proteolysis run concurrently at 30°C. but not at 32° or 37°C.[181,536] Both endo-, and extracellular proteinases have been isolated from lactic streptococci.[498,499,537] Proteolysis in milk at 32°C. is detectable within 4 hr.

Suggesting the importance of proteolysis in starters is the observation that various cultures under conditions of identical acid development (0.55%) produced curd with different curd tension values.[196] The variation may be attributed directly to variations in proteolysis,

or indirectly to differences in buffering action brought about by different degrees of proteolysis.[536]

Free amino nitrogen is not associated with bitterness. Several investigators believe that the development of bitterness indicates a deficiency in enzymes required to hydrolyze the bitter primary degradation products of cheese proteins.[109]

Proteolysis by L. casei.—Proteolysis develops more rapidly in Cheddar cheese inoculated with *L. casei* than in control cheese.[277] In some instances the difference is evident throughout the ripening period, in others after 8.5 months.[46] Proteolytic strains added to Cheddar cheese encouraged flavor development and improved flavor in mature cheese.[550]

Proteinases isolated from disrupted cells exhibited maximal activity at 15° and 38°C., and at a pH near neutrality.[13] Brandsaeter and Nelson[33,34] attribute maximum activity at pH 7.0 to a peptidase, and maximum activity in the pH range 5.5–6.5 to a proteinase.

Stereospecific deaminases in *L. casei* brought about deamination of serine at pH 5.4 and 8.1, and asparagine and threonine at pH 8.1.

Two deaminases appear to be involved. Thus deamination of DL-serine proceeded equally well at 52°C. and pH 7.0 as at 46°C. and pH 4.6.[269–271]

Proteolytic activity is exhibited by some strains of *Lactobacillus brevis*,[84] *L. bulgaricus*,[546,547] *Lactobacillus helveticus*,[6A] and *L. lactis*.[31] The subject has been reviewed by Marth.[327]

Proteolysis with Micrococci.—Micrococci comprise 78% of the nonlactic bacteria in Cheddar cheese.[2] The proteolytic apparatus of *Micrococcus freudenreichii* functions optimally at 30°C. and at a pH near neutrality.[13] Analysis of the several sets of conditions for the optimal activity of one year old Cheddar cheese proteinases support the view that micrococci may contribute to the total proteolytic activity.

Proteolysis by B. linens.—An extracellular proteinase isolated from a two day old culture of *B. linens* in a liquid medium was active over a pH range between 5.6 and 8.8 and exhibited maximum activity at pH 7.2–7.3.[125] Proteolysis was detectable at 0° and 60°C. and optimal at 38°C. The enzyme hydrolyzed α and β-casein but not β-lactoglobulin and α-lactalbumin. No evidence was found of polypeptidase and dipeptidase activity.[486] Free amino acids are liberated in Limburger cheese inoculated with *B. linens*[492] suggesting perhaps that under certain conditions enzymes other than proteinases are secreted by the organism which occurs in the flora of surface-

ripened cheese and which it is believed contribute to their characteristic body and flavor.[126]

Proteolytic Action by Molds.—Blue cheese with inadequate growth of *Penicillium roqueforti* reflecting inadequate proteolysis is tough and crumbly instead of soft and smooth.[169] The proteinase of *P. roqueforti* is of the trypsin type with an optimum activity between pH 5.8 and 6.3 and is not inhibited by NaCl at levels found in blue cheese.[169] Proteinases and related enzymes in *Penicillium camemberti* are chiefly responsible for proteolysis observed in Camembert cheese.[169]

Cultured Milks

The effect of bacteria on the flavor and consistency of milk was utilized in making special milk drinks many years before bacteria were seen by van Leeuwenhoek. Most of these fermented drinks originated in southern Russia and in the countries around the eastern end of the Mediterranean Sea. They were made by producing conditions in milk favorable to the desired fermentation, or by inoculating milk with small amounts of prepared milk. Usually the unwashed containers were depended upon to provide the starter flora, especially when skin bottles were used. Under these conditions a mixture of bacteria was present, and flavors were produced which were difficult to reproduce with pure cultures, although the essential bacteria of these fermented drinks were eventually known. In all instances the basic fermentation is a lactic one. Sometimes it occurs in combination with the production of gas, a mild alcohol fermentation, and some proteolysis.[79] Yoghurt is said to contain about 0.2% alcohol,[79] and kefir may contain about 1.2%. Some fermented drinks can be made from nonfat dry milk solids and water, and the body and texture of such a reconstituted milk can be improved by the addition of 0.35% gelatin.

One of the more popular fermented milks is known in Bulgaria and Turkey as yoghurt or yaourt. Alternately it is known in Armenia as matzoon and in Egypt as leben. In this milk, the fermentation is brought about entirely by lactic acid bacteria, including *Thermobacterium yoghurt* of Orla-Jensen (probably *L. bulgaricus*), *L. bulgaricus*, and *S. thermophilus*. *S. lactis* may be present but is not essential; *L. acidophilus* is sometimes added, in which case the product is called yoghurt acidophilus milk. The fresh milk is pasteurized, cooled to 40°–45°C., inoculated, and held at that temperature until ready for use; it is then refrigerated. Rosell[439] recommends an incubation temperature of 45° to 48°C. At this temperature, 3.5% acid devel-

ops in 24 to 36 hr. The body of yoghurt may be improved by heating the fresh milk at 90°C. for 5 min., homogenizing the milk after the culture is added, and adding spray-dried milk (3 parts per 100). Yoghurt is made from cow's milk, either pasteurized or partially evaporated, and in some places from sheep or buffalo milk. The Egyptian leben is said to contain a lactose-fermenting yeast which produces a mild alcohol fermentation. Winckel[538] has described two new fermented milks called kajobst and kajovit, which undergo a modified yoghurt fermentation.

Modern yoghurt is almost invariably made from a mixed culture of *S. thermophilus* and *L. bulgaricus*. The two cultures are maintained separately.

Schulz *et al.*[454,455] identified acetaldehyde as the compound responsible for the characteristic flavor and aroma of yoghurt milk. The addition of 0.001 to 0.005% acetaldehyde to milk soured with *S. thermophilus* effected a yoghurt-like aroma and taste in the product. Four ranges of color intensity were defined, and milks were grouped accordingly. Sour milks possessing well-developed yoghurt flavor and aroma contained a relatively high concentration of acetaldehyde, and those lacking these qualities contained less.

The popular American cultured milk is buttermilk. In making cultured buttermilk commercially with *L. bulgaricus*, it is desirable to temper the sharp acid flavor produced by the bulgaricus culture by growing a streptococcus in association with it. However, the maintenance of the two in mixed culture is difficult; the lactic streptococci cease growing in the pH range 4.0–4.2, whereas *L. bulgaricus* tends to decrease the pH considerably below this level. The desired end may be accomplished by separate growth in separate containers, followed by proportioning and mixing to give a product with the desired flavor and texture. The product made with *L. bulgaricus* is known today as Bulgarian buttermilk. A more popular beverage employs either *S. lactis* or *S. cremoris* grown together with either *L. dextranicum* or *L. citrovorum*. One function of the streptococcus is to provide a sufficiently acid environment for the "aroma" compounds to be produced maximally.[247,348] If the starter culture is permitted to develop 0.80–0.85% acidity before it is used, and if the incubation temperature is 21°C., associative growth will produce a buttermilk with excellent flavor and body characteristics.

Kefir is usually made from cow's milk, and is peculiar in that the fermentation is brought about by kefir "grains," which resemble miniature cauliflowers. These "grains" consist of casein, yeasts, and bacteria. The microorganisms include lactose-fermenting *Torula*

yeasts, *S. lactis*, *Betabacterium caucasicum* (probably an *L. brevis* variant), and glycogen-containing rod-shaped kefir bacilli (Henneberg). The grains increase in size in the fermenting milk, and may be strained out, dried, kept for long periods, and used as inocula. The fermentation is usually carried on in closed bottles so that gas is retained and the milk becomes effervescent.

In Russia a milk drink made from unpasteurized mare's milk, and known as kumys, is used extensively. The fermentation is caused principally by *L. bulgaricus*, lactose-fermenting *Torula* yeasts, and *L. leichmannii*.

Kuban fermented milk, a product of southern Russia, is made from pasteurized milk by a combined lactic and alcohol fermentation. The microflora include a lactic streptococcus resembling *S. lactis* var *hollandicus*, a lactic rod of the *L. bulgaricus* type, and three yeast types.

Taette milk is a sour milk used in the Scandinavian Peninsula. A slime-producing fermentation is induced by a variant of *S. lactis* to which the name *S. lactis* var. *taette* has been given. This is possibly identical with *S. lactis* var. *hollandicus*, which has been used in starter to make Edam cheese, and with other streptococci associated with "ropy" milk.

A milk drink known as saya is prepared from fresh unheated milk, ripened at first by *S. lactis* and later by a lactobacillus. In saya milk, considerable carbon dioxide, and vigorous proteolysis, are produced. Characteristic of the fermentation is a six-day ripening period at 11°C. Corminboeuf[79] has described numerous milk beverages, including mazun, groddu, skorup, and tättemjolk.

Undesirable Fermentations in Milk

The flavor and body of cultured milks and milk products are distinguished by a delicate balance between the constituents of the cultured product. Unless conditions of culture are carefully controlled, this balance may not be achieved even when pure cultures are employed. The empirical formulations relating to the proper cultural and external conditions constitute the art of fermentations. Apart from defects arising through use of improper conditions of culture, a series of defects may arise in milk and milk products because of the contamination of a milk supply by unwanted organisms, and because of the tendency of many organisms required in fermentations to produce antibiotics.

Flavor defects have been characterized as bitter[314,490] (see section on proteolysis), rancid, ester,[82] cresol-like,[334] barny,[318] doughy,[447]

fruity,[209,424] fishy,[163] malty,[171] potato-like,[35] turnip-like,[286] caramel,[240,446] cabbagy,[115] metallic,[9] putrid,[389] and feed.[442,443] Body defects such as sweet curdling in evaporated milk, gassiness in cheese and evaporated and sweetened condensed milk may be brought about by bacterial contaminants. A body defect of rather wide occurrence, which may reach epidemic proportions, is ropy or slimy milk.

Ropy and Slimy Milk.—Ropy or slimy milk of bacterial origin becomes apparent only after the milk has been held for several hours, and is therefore distinguishable from the stringy milk associated with mastitis.[110,178] The ropiness may be evident only as a slightly abnormal viscosity, or it may be so pronounced that the affected milk may be drawn out in fine threads a yard long, and in some instances may assume a gel-like consistency. Thickening may be confined to the milk's top layer.

Among the bacteria causing ropiness are active gelatin liquefiers, including some of the hay bacillus type. More frequently, however, epidemics of ropy milk are caused by some members of the coliform group or the lactic streptococci. The common occurrence of the defect in the presence of certain streptococci has led to the assignment of distinguishing names to these. *S. lactis* var. *taette*, *S. lactis* var. *hollandicus*, and varieties of the common *S. lactis* are the essential organisms in Swedish ropy milk and in certain Edam cheese starter cultures. Among the organisms associated with the development of ropiness are these: *Alcaligenes viscolactis*,[172,202,203,333,448] *S. lactis* var. *hollandicus*,[165,170] certain corynebacteria,[203] and some organisms of the *Escherichia-Aerobacter* group.[448,450] Ordinary milk streptococci, such as *S. lactis*, *S. cremoris*, or *S. thermophilus*, may at times cause ropiness.[203] Certain strains of streptococci, according to Hammer, acquire and lose quite easily their ability to produce ropiness. Rope-producing strains are more oxygen-exacting than nonrope-producing ones and develop less volatile acid. The induction of rope-producing properties in bacteria by means of bacteriophage has been observed.

The flavor of ropy milk, unless the effect is associated with a lactic fermentation, is indistinguishable from that of normal milk; nor is the milk unwholesome in any way.

The immediate cause of the ropy or slimy condition is the bacterial formation of gums or mucins. The gums are the more common cause. These are probably galactans produced by the fermentation of lactose. Some of the active peptonizing bacteria produce sliminess by the formation of mucins, which are combinations of proteins with a carbohydrate radical. The development of sliminess is closely associated with capsule formation.[42,165] The ability to pro-

duce slime in milk is rather general among bacteria and is readily acquired and lost. Epidemics are caused by some members of the *A. aerogenes* group or the lactic streptococci.

Emmerling,[108] in 1900, and Schardinger,[452] in 1902, observed that *Aerobacter aerogenes* produced slime in milk. The empirical formula $(C_6H_{10}O_5)_n$ was assigned to the slimy substance. It dissolved readily in water, yielding a gelatinous solution; it was optically inactive and was non-reducing toward Fehling's solution. Hydrolysis with dilute acids yielded a reducing sugar; oxidation with nitric acid yielded both mucic and oxalic acids. The gummy substance was called arabogalactan. The galactan-producing properties of *Bacterium sacchari* and *Bacterium atherstoni* were studied by Smith,[463] who, upon hydrolyzing the product, obtained galactose and arabinose and upon oxidation obtained mucic and oxalic acids.

Antibiotic Production in Milk

Production of antibiotic-like substances in cultured dairy products has been associated with the homo-, and heterolactic streptococci, some of the lactobacilli and *B. linens*. Production may be unwanted and fortuitous, as in commercial starter cultures or it may be desired and encouraged.

Nisin.—Elaboration by *S. lactis* of a substance inhibitory to *L. bulgaricus* was reported by Rogers[436] in 1928. The substance named "nisin" produced by some strains of *S. lactis* is a large polypeptide with a molecular weight of approximately 10,000.[336] Lanthionine and a structural isomer of cystathionine, two sulfur containing amino acids were recovered from hydrolysates by Newton *et al.* who concluded that nisin resembled the antibiotic subtilin.[374] A partially purified preparation (mol. wt.—7,000) by Cheeseman and Berridge lacked amino or carboxyl end groups, but contained side chains with the epsilon amino group of lysine and the imino group of histidine.[61] Baribo and Foster observed that an endocellular inhibitory substance (probably nisin) was liberated when cultures of *S. lactis* were acidified.[8] Cultures boiled or autoclaved for 10 min. at pH 4.8 retained their activity, whereas those heated at pH 7.4 rapidly lost about 50%.

Nisin dissolves in aqueous solution at pH 7, 5.6, and 4.2 to the extent of 75, 1,000 and 12,000 μg. per ml. respectively.[189] Solubility is substrate dependent. Nisin assays are based on its inhibitory action toward *Streptococcus agalactiae* in a tube dilution test.[205,335] One Reading unit is defined as that amount of antibiotic preparation dissolved in N/20 HCl which gives the same inhibition as a standard

preparation. A 0.1% solution of the standard contained 10,000 Reading units, i.e., it was inhibitory at the 1:10,000 dilution. Modified tests using litmus milk and *S. cremoris*,[131] and the one-hour resazurin test and *S. cremoris*[125] have been described. Modified microbiological assays have been reported by a number of workers.[21,325,485] Nisin is distinguished from chemical preservatives by means of its antibacterial spectrum particularly with respect to its activity toward some yeasts and lactobacilli.[83] Paper chromatography is useful for identification purposes.[464]

Antibacterial Spectrum.—Hawley reported that various species and strains of the genera *Staphylococcus*, *Streptococcus*, *Neisseria*, *Bacillus*, *Clostridium*, and *Corynebacterium* are inhibited by nisin.[189] Mattick and Hirsch added actinomycetes, pneumococci, mycobacteria, and *Erysipelothrix* to this list.[335] The nisin concentration required for complete inhibition is organism specific and ranges from 0.25 to 500 units per ml. Inhibition of *L. casei* by antibiotics from *S. lactis* and *S. cremoris* was observed by Baribo and Foster.[12] Inhibition by nisin of *Propionibacterium* but not coliform bacteria was reported by Galesloot.[130]

Nisin Inactivation.—*L. plantarum* isolates (thermal death time, 5 min. at 65°C.) from milk and cheese reduced nisin activity in these substrates.[255] *S. faecalis* and *S. lactis* isolates from raw milk destroyed nisin.[129] Galesloot observed that the use of nisin-producing starters in cheese manufacture is uninviting if large numbers of group N streptococci are present.[129] Galesloot also observed that some cultures of *Leuconostoc* were antagonistic. Nisin (but not subtilin) destroying nisinase, an enzyme, has been recovered from some strains of *S. thermophilus*.[3]

Relationship between Starter Cultures and Streptococcal Antibiotics.—When antibiotic-producing and nonproducing strains of *S. lactis* and *S. cremoris* from commercial starter cultures were mixed in equal proportions, the antibiotic producing strains soon predominated.[219] Domination occurred in only a day or two.[71] Emergence of a predominant strain may be accompanied by a loss in starter activity, and certainly renders the starter more susceptible to complete inactivation by bacteriophage.

Heterolactic Streptococci.—Ritter in 1945 noted that two strains of *S. lactis* were inhibited by five strains of betacocci (*Leuconostoc* sp.) when grown at 20°C.[430] The subject has been approached recently with renewed interest. A creaming mix made up in part of skimmilk cultured with *L. citrovorum* when added to cottage cheese inhibited such spoilage organisms as *P. fragi*, *Pseudomonas*

putrefaciens or coliforms but not the yeasts *G. candidum* and *Candida pseudotropicalis.*[332] Marth and Hussong showed that the filtrates from cultured skimmilk, in which four strains of *L. citrovorum* were allowed to ferment citrate, inhibited to different degrees *S. aureus, A. aerogenes, A. viscolactis, E. coli, P. fragi,* and *P. fluorescens,* but in no instance the yeasts *Torula glutinis, S. cerevisiae, Saccharomyces fragilis,* or *Mycotorula lipolytica.*[329] Dilution of the filtrates to the level at which they might be present in cottage cheese eliminated the inhibition of all bacteria except one of two strains of *P. fragi* and 1 of 5 strains of *P. fluorescens.*[328] Collins noted that 3 of 6 strains of *S. diacetilactis* evaluated formed an antibacterial substance similar to that produced by *S. cremoris.*[71] Some strains of *S. lactis, S. cremoris,* and *S. diacetilactis* were inhibited.

These observations suggest that care must be exercised to combine only suitable strains in the compounding of a mixed strain starter culture.

Lactobacilli.—Kodama[250] isolated an antimicrobial substance designated "lactolin" from *L. plantarum,* and Wheater,[529] a substance designated as "lactobacillin" from organisms resembling *L. helveticus.* "Lactobacillin" inhibited *C. butyricum* in Gruyere cheese.[208] Cultures and a derived filtrate of *L. helveticus* reduced gas formation by *E. coli*[340] and inhibited *P. shermanii* and other propionibacteria.[539] Pasteurized cultures and culture filtrates of *L. acidophilus* inhibited growth of *E. coli.*[390] Concentrated sterile culture filtrates prevented, and growing *L. acidophilus* cultures halted development of *E. coli, P. fluorescens, Shigella* sp., *Salmonella* sp., and aerobic spore-forming bacilli.[399] "Lactocidin" the antibiotic produced by *L. acidophilus* was isolated by Vincent *et al.*[502] It has a wide spectrum of antibiotic activity. Winkler reported on the inhibition of propionibacteria by milk cultures of *L. acidophilus.*[539] Some lactobacilli, according to DeKlerk and Coetzer, produce substances inhibitory to other bacteria of the same genus.[91]

Antibiotics in Cultured Milks.—Some cultured milks exhibit antibiotic activity, the causative organisms for which are obscure. Thus acidophilus milk is antagonistic to *E. coli* and bactericidal to *Mycobacterium tuberculosis;*[46] yoghurt inhibits *Erysipelothrix rhusiopathiae,*[238] *E. coli,*[237] and human, bovine and BCG strains of *M. tuberculosis;*[480] kumys is bacteriostatic or bactericidal to *E. coli, S. aureus, B. subtilis, B. cereus, A. aerogenes,* and other organisms;[45,460] kefir is inhibitory to *E. coli, S. aureus,* and *B. subtilis;*[45] and "kuränga" is inhibitory to mycobacteria and organisms in the genus *Bacillus.*[62]

Antibiotics from Surface Ripened Cheese.—An antimicrobial agent attributed to *B. linens* reportedly appears in surface-ripened cheese stored at 2–4°C. for 8 wk., and is inhibitory to *S. aureus*, *B. cereus*, and *Clostridium botulinum*. Strains of *B. linens* yielded culture fluids inhibitory to the germination and outgrowth of *C. botulinum* type A. spores.[142,144] Organisms other than *B. linens* on the surface of cheese contribute minor antimicrobial activity.[143] The inhibitory substance from *B. linens* withstood heating at 121°C. for 25 minutes.[144] Its properties differed from those of nisin.[143]

INDUSTRIAL FERMENTATIONS WITH LACTEAL MEDIA

In the realm of purely industrial fermentations, milk and its derived products have not, for historic and economic reasons, received their full share of attention. Decentralization of industry in the early days of the manufacture of casein and cheese weakened the competitive position of the low-solids by-product, whey, relative to that of grains and molasses. With changing economic and market trends, it is to be expected that the by-products of milk, produced at a rate of more than 12 billion lb. annually, and which are intrinsically suited for many if not all industrial fermentations will occupy a strengthened competitive position. In times of unusual demand, such as wars produce, these by-products have been shown to possess a strong industrial potential.

Whey has been and is being used in the manufacture of lactic acid. Lactose has been and still is the carbohydrate of choice in the manufacture of penicillin. Whey has been used on a large scale in the microbiological synthesis of riboflavin and in the concomitant production of butanol and acetone. The utilization of whey in the manufacture of alcohol, yeast and fat has been studied. Whey is a suitable substrate for the microbiological synthesis of vitamin B_{12} in a number of fermentations. Enzymatic digests of casein are used extensively in the manufacture of antibiotics, and lend themselves to any fermentation, such as the propionic acid one, which requires a degraded source of amino acids. Casein and the nitrogenous constituents of whey give rise to large yields of riboflavin in flavinogenesis by means of the fungus *Eremothecium ashbyii*.

Skimmilk is recommended as a medium for the microbiological synthesis of the antibiotic, nisin.

Bacterial oxidations may yield useful products. Vinegar and vinegar substitutes may be obtained from whey in acetic acid fermentations. Lactobionic acid may be obtained in high yields by

the action of *Pseudomonas graveolens* on the lactose in whey. Uses of fermented whey as a food or beverage are known or have been suggested.

Production of Lactic Acid

The microbiological production of lactic acid utilizing whey is industrially important. In this fermentation the culture of choice is *L. bulgaricus*, for a number of reasons: (1) it is homofermentative, producing almost theoretical yields of lactic acid; (2) it is thermophilic and, having an optimum growth temperature between 45° and 50°C., it can be grown in pasteurized rather than sterile media without much danger that the medium will become contaminated; (3) it is acid-tolerant and, in a batch process, periodic neutralization of the medium is required with relative infrequency; and finally (4) it grows under either aerobic or anaerobic conditions.

The fermentation with *L. bulgaricus* is apt to be sluggish in whey, and hence it is grown in association with a yeast (*Mycoderma*). The function of the yeast is not clearly defined; it may be presumed that it produces stimulatory growth factors for *L. bulgaricus*—an organism which is highly fastidious in its nutritional requirements. In this connection it should be borne in mind that certain strains of *L. bulgaricus* are unusual in that they cannot use the pyrimidine derivative uracil, but instead require orotic acid; nor can the species in general use pantothenic acid, but instead requires pantetheine; and finally, the species cannot utilize biotin, but instead requires such unsaturated fatty acids as oleic or linoleic. In connection with mineral requirements, *L. bulgaricus*, like other species in this genus, probably requires for maximum growth rates manganese in excess of the quantity usually present in milk. The fermentation proper has been described in some detail.[49,387]

The Butanol-Acetone-Riboflavin Fermentation

In addition to volatile solvents, fermentations with *C. acetobutylicum* may yield the important vitamin, riboflavin, in significant quantities. Optimal quantities of iron between 1.5–2.0 ppm.,[339] and either certain salts of organic acids or calcium carbonate[548,549] are required for maximal synthesis of riboflavin, and maximum fermentation rates.

Leviton,[294] working with whey and with a semisynthetic medium, studied the stimulatory effect of calcium carbonate on the one hand and the inhibitory effect of iron on the other. The action of iron was not only inhibitory but also destroyed any riboflavin added to the

medium. A parallelism existed between the catalytic effect of ferrous ion in the chemical reaction between riboflavin and hydrogen peroxide and the destructive action of iron *in vivo*. Furthermore, the inhibitors of the chemical reaction were inhibitors of the destructive microbiological reaction, and this phenomenon was particularly striking with the specific inhibitor catalase. Iron tolerance in the microbiological synthesis was increased in the presence of catalase and other inhibitors; the maximal rate of fermentation, even in the presence of suboptimal concentrations of iron, could be augmented to the point at which it equaled that of a normal maize fermentation. It appeared that the inhibitory mechanism in flavinogenesis involved the production of small quantities of hydrogen peroxide. Evidence for the accumulation of hydrogen peroxide by an obligate anaerobe *C. kluyveri* during the oxidation of butyrate has been reported recently.[303]

The stimulatory effect of calcium carbonate was shared by a large number of sodium salts of organic acids, such as acetic, lactic, pyruvic, and citric.[295] In their absence, the fermentation was characterized by two lag phases, one occurring at the onset of fermentation, and the other, rather long in duration, occurring later at the so-called "break" in the fermentation. Furthermore, in the absence of these salts, no riboflavin was synthesized, and only relatively small quantities of butanol were formed. In a rich fermentation in which a large quantity of riboflavin was produced, there was some indication of lysis. Lysis was caused by butanol and was a concomitant of good yields of riboflavin. By adding butanol in small concentrations to an active fermentation medium deficient in asparagine, good yields of riboflavin were obtained, even though the deficiency in asparagine conduced to an "acid" fermentation (one in which solvent yields are low).

Thus lysis was a concomitant of good yields of microbiologically synthesized riboflavin, butanol was the instrumentality for lysis, and added butanol in so-called "acid" fermentation promoted riboflavin synthesis.

The butanol-acetone fermentation, utilizing whey supplemented with yeast extract, is substantially a butanol fermentation. Approximately 80% of the volatile solvents produced thereby is butanol, 13% is acetone, and 5% is ethanol. Approximately 30% of the lactose fermented is converted to butanol, 5% to acetone, 2% to ethanol, and the balance largely to CO_2 and small quantities of butyric and acetic acids, acetylmethylcarbinol, and hydrogen.[295]

C. acetobutylicum is not too exacting in its growth requirements.

Asparagine is needed in order to effect the normal production of solvents in what otherwise would be an acid fermentation.[483] Both biotin and *p*-aminobenzoic acid are required in trace quantities, 0.001 and 0.05 μg. per ml., respectively. Iron is essential in minimal but variable quantities for attainment of maximal fermentation rates.[294–296,339] The requirement for iron varies with the composition of the medium; potassium is also required.[87,88] Trace quantities of manganese sulfate, lithium chloride, strontium chloride, tin chloride, and zinc chloride aid the fermentation in whey.[339]

Leviton and Burkey[299] added yeast extract, liver extract, or cornmeal to whey, thus achieving a normal fermentation and avoiding the addition of iron. The presence of these added solids to the extent of one percent in whey assures a normal fermentation and high yields of riboflavin.

The physiological state of the organism influences the yield of riboflavin, as well as that of volatile solvent, and consequently requires control.[294] Transfer of inocula at a time when approximately 25% of the gaseous products of fermentation had evolved is conducive to high yields.

Production of Alcohol

Although lactose-fermenting yeasts have been available for some time, it was not until recently that their possible utilization in the production of ethyl alcohol and yeast from whey solids received serious study. Browne[41] observed that certain *Torula* species yielded more alcohol than might have been expected from statements in the literature. Working with four kefir yeasts, two *Torula* species, one of *Torulopsis*, and one additional yeast species, he obtained alcohol in yields 68 to 80% of theoretical. A maximal yield, 80.3%, based on a theoretical yield of four moles alcohol per mole lactose fermented, was obtained with a strain of *Torula cremoris* in a 21.7-hr. fermentation at 30–32°C.

Continuing Browne's work, Rogosa *et al.*[438] extended the scale of operation, and employed in addition to *Torula* species, *S. fragilis*, *Saccharomyces lactis*, *Saccharomyces anamensis*, *Zygosaccharomyces lactis*, *Mycotorula lactis*, and *C. pseudotropicalis*. Again *T. cremoris* gave the highest yields. The yield of alcohol averaged 90.73% under laboratory conditions and 84% under pilot-plant conditions.

Alcoholic Beverages

Whey supplemented with malt wort has been used as a raw material for the preparation of beer. Dietrich[97] added 5.4% malt wort to

dilute whey (2.5% solids), precipitated the albumin at 90°C., and filtered the mixture. He then inoculated the filtrate with a strain of the yeast *S. lactis*, and after 5–7 days obtained a product with a true beer taste and character.

Whey may be fortified with sucrose, and fermented with yeast to yield an alcoholic whey. Upon freeze concentration a whey liquor with 10 to 69% alcohol was obtained.[112] The alcohol fermentation carried out in whey supplemented with brown sugar yields a whey cordial.[10]

Microbiological Fat Synthesis

Limiting Factors.—Pioneering research brought about the discovery that an ample and sometimes a critical supply of oxygen was necessary.[306] It was incorrectly assumed, however, that only in surface culture—a condition more suited for the growth of fungi than for the proliferation of yeast—could the necessary supply of oxygen be maintained.[304–307]

Fat was synthesized only within a definite pH range which varied with the medium and the type of organism.[25,428] A high concentration of assimilable carbohydrate and a relatively low concentration of water conduced to the proliferation of fat-rich cells.[25] A small inoculum, and usually incubation temperatures, at the optimum for growth or slightly under, were beneficial. In any given culture, the ratio between cellular fat and protein increased with age. Of the utmost importance was the observation that nitrogen, as in plant nutrition, was a nutritive requirement for fat synthesis[193,414,453] and since this requirement and that of a high sugar concentration were less exacting for molds than for yeast growth, much of the early research work on fat synthesis was devoted to the study of molds.

Inverse relationships were found to apply between the fat coefficient on the one hand and the conversion, protein, and carbohydrate coefficient on the other.[39,195,414] Responding to a nitrogen deficiency both yeasts and molds synthesized more fat, and less protein and cellular materials. Environmental factors which were favorable to the synthesis of fat were precisely those which were antagonistic to the synthesis of protein or carbohydrate.

Fat Synthesis by Yeasts.—The studies with yeasts particularly those with *Endomyces vernalis* showed that commercially acceptable yields of fat could be obtained microbiologically.[307] Processes based on surface culture were too costly, however, and therefore submerged techniques were sought. Nilsson, Enebo, and coworkers[111,376] working with *Rhodotorula glutinis, Rhodotorula gracilis,*

and synthetic media containing invert sugar, pH 4.0–4.7, and employing techniques similar to those employed in compressed yeast manufacture obtained yields of dried *R. glutinis* yeast after 3–4 days amounting to 31–36% of the sugar consumed, and gratifyingly, it contained 25–30% fat. Results with *R. gracilis* were even more encouraging. Schulze[456] pursued the problem of producing "fat" yeast on a commercial scale using continuous methods with amazing success. He first showed that not only under conditions of nitrogen deficiency but also under conditions of phosphorous depletion, fat-rich yeasts could be obtained, and moreover, he found that proliferation of yeast, slow in nitrogen deficient medium proceeded more vigorously in phosphorus deficient ones.

Starting with a culture of normal composition isolated from sulfite waste liquor, Schulze, in the course of 3–4 wk. of continuous cultivation, obtained a culture containing chiefly fat-rich cells. With this as an inoculum and processing 12,000 liters phosphorus deficient liquor daily he obtained 150 kg. dried yeast per day of which 20–23% was fat, and 25–30% protein. The yield based on added reducing substances was approximately 40%, and the generation time was 10–12 hr. In one experiment 90,000 liters of waste liquor was processed daily. These experiments constitute the first attempt to produce "fat" yeasts on a commercial scale.

Fat Production in Whey Media.—Whey is a suitable medium for the proliferation of fat-rich fungi, and an excellent sulfite waste liquor supplement for the growth of fat-rich yeasts. Considerations of economy apart, there is some question whether whey is sufficiently deficient in phosphorus to support the propagation in continuous culture of fat-rich yeasts. Milk serum and hence whey contains about 1.4 gm. P_2O_5 per liter.[7] Yeast will proliferate normally in a medium containing a steady state concentration of 0.7–0.8 gm. P_2O_5 per liter.[456] Hence the P_2O_5 in whey would support a normal, rather than the abnormal, sugar assimilation necessary for fat biosynthesis. However, the possibility suggests itself that calcium or magnesium supplements could be employed to lower the concentration of diffusible phosphate. Thus it has been found in some experiments that added calcium salts conduce to high fat yields,[254] and this may perhaps be related to suppression of phosphate ions.

Whey has been found to be superior to other natural media for the growth of fat-rich species of the genus *Geotrichum*. Fink and his coworkers found only two strains among many which had the capacity to synthesize fat to a commercially significant degree. These strains of *G. candidum* were distinguished by certain morphological charac-

teristics—a yellow-white convoluted structure of the mat, as compared to the snow-white silk-like texture found in low-fat strains.[117] Almost simultaneously Geffers carried out similar screening tests with the interesting result that only the good producers of fat assimilated lactose.[135]

G. candidum is found in abundance in dairy products and is always found in Camembert cheese. Unsupplemented whey obviously is a suitable medium. However, fortification with 0.05% KH_2PO_4, 0.02% $MgSO_4$, and 0.1% $(NH_4)_2\ SO_4$ has been recommended. Fink and his co-workers obtained 5.7 gm. dry material after incubating fortified whey six days in Jena culture flasks.

The dry substance contained 17.6% crude protein, and 22.5% crude fat. After 7 days the yield of cellular material began to decrease from its maximum of 6.1 gm. until at the end of 16 days it had declined to 5.7 gm. The per cent of raw protein increased and that of fat decreased during this late period, whereas during the first 7 days, the relationship prevailing between protein and fat had a diametrically opposite character. The pH of the medium originally 4.2 rose after 2 days to 5.0, remained at this value for the next 5 days, and then rose during the final 9 days to 8.1. The fat had a pale yellow to pale brown color. Of the two strains studied, one yielded a product with a vaseline-like consistency, and the other, fat which was liquid at room temperature. The fat characteristics were not only dependent on strain, but also on conditions of culture.

Advantages accruing to the use of mixed cultures were reported by Fredholm[123] who observed that symbiosis of *G. candidum* and various lactic acid bacteria such as *S. lactis*, *S. cremoris*, and *L. citrovorum* yielded 44.7 gm. dry cells (42% fat) per 100 gm. lactose plus lactic acid compared with 47.2 gm. (25.5% fat) obtained in control experiments. Inoculation with lactic acid bacteria followed by 40 hr. inoculation with *G. candidum*.

Xylose wort as a basal medium for fat synthesis by *Candida reukauffii* requires supplementation with extracts and mineral salts.[427] Whey was found to be an exceptionally good supplement up to concentrations of 10% in a basal medium (pH 6.8) containing about 2% xylose, 0.2% K_2HPO_4, 0.2% K_2SO_4, 0.05% $MgSO_4$, 0.005% $FeSO_4$, and 0.012% $(NH_4)_2HPO_4$ and 1% wheat bran extract. The addition of 10% whey effected an increase in dry yeast production from 3.60 to 10.42 gm. per liter, an increase of fat from 0.62 to 2.57 gm. per liter, and an increase of protein from 0.62 to 1.05 gm. per liter. Testifying to the efficient utilization of sugar in the presence of whey were the exceptionally high conversion and fat coefficients of 55.7 and

13.76 respectively. Considering that the fat coefficient approached quite closely the theoretical maximum of 15, and that such high yields were obtained in 48 hr. with an inoculum of only 0.1 gm. dry cells per liter, one may view the results obtained by Rippel-Baldes with satisfaction. In experiments with media containing progressively increasing amounts of sugar it was observed that all three coefficients—fat, protein, and conversion decreased progressively contrary to the expected inverse relationships between the coefficients. However, if sufficient time was allowed for nearly all of the sugar to be consumed the anticipated relationships were observed.

The yeast oil had the following characteristic: consistency, fluid; sp. gr., 0.921; unsaponifiable matter, 0.21; iodine No., 70; saponification No., 197; and in these characteristics the oil approximated those associated with olive oil and the oils obtained with *E. vernalis* and *Oospora* species.

Fat Synthesis in Whey with Molds and Mold-like Fungi.—Geffers working with unsupplemented whey and with a selected strain of *Oospora wallroth* obtained 3–5 kg. fat and 10–12 kg. dry substance from 1,000 liters of whey after a lengthy incubation.[135]

Schulze[456] working with whey and a species of *Trichosporon* (synonymous with *Oospora moniliaformis*) obtained after 5 days incubation at 24°C. 47.6 gm. dry substance per 100 gm. sugar, 12.4 gm. crude fat, and 7.6 gm. crude protein.

Recently Wix and Woodbine grew *Aspergillus ustus* in whey fortified with 1.14 gm./l. NH_4NO_3. Incubated on a shaker, the mold used up 96% of the lactose yielding 17 gm. mycelial felt per l. with the composition 13% protein and 28% fat.[539A] Results with *Penicillium frequentans* were not encouraging.

Lipid Composition.—The higher fatty acids predominate in the fat from both yeasts and molds, and the unsaturated predominate over the saturated acids. Thus *Penicillium javanicum* reportedly contains 60.8% and 30.8% unsaturated and saturated acids respectively,[517] *G. candidum* 53.1 and 42.8% respectively,[239] and yeast-fat 50.5 and 15.4% respectively.[484] Palmitic and stearic acids are the chief saturated acids, and oleic acid is the chief unsaturated acid. The latter was found in fat from yeast, *P. javanicum* and *G. candidum* to the respective extents of 47.6, 31.7, and 41.2%, and linoleic acid was found to the extent of 2.9, 29.1, and 11.4%. Linolenic acid was not found in yeast-fat, but occurred in samples from *G. candidum* to the extent of 0.12%.

Astonishingly large amounts of phosphatides in yeast grown under controlled conditions have been reported. Of the 2.87% total fat in

beer yeast, Salisbury and Anderson found that 58% consisted of phosphatides.[449]

Yeast fat is an excellent natural source for ergosterol and related sterols.[533,534] Ergosterol is also found in the fat from *P. javanicum*. Characteristic of the unsaponifiable fraction of yeast fat is its rather high concentration of the unsaturated hydrocarbon, squalene.[484] Certain yeasts, especially the red pigmented ones, abound in carotenoids.[59,119,319]

Squalene biogenesis has been followed in model systems containing yeast enzymes.[322] The composition of the lipid fraction of microorganisms varies with differences in conditions of culture. Thus, the solidification point of fat from *P. javanicum* depends on the sugar concentration of the medium upon which the mold was grown.[518] Fat characteristics such as melting point, iodine number and average molecular weight as well as the concentration of unsaponifiables may vary with changes in the composition of the medium (activity and source of nitrogen) in which the microorganism is grown.[393,411,412] Much work has been reported on factors influencing sterol syntheses by yeasts and fungi.[160,331] Klein[245,246] working with resting cells of anaerobically developed *S. cerevisiae* observed an increase in cellular sterols from 2 to 5 mg. per gm. dry cells to 15 mg. when the culture was aerated in a glucose-phosphate buffer medium. Acetate was diverted to sterol synthesis in the absence of CO_2 but in the presence of CO_2, a five-fold increase in fat synthesis occurred.

Production of Yeast

The conversion of lactose into edible protein both for animal and human consumption is an inviting prospect especially in view of modern trends in nutrition which emphasize the relative importance of protein in rations. The principles underlying the microbiological conversion have been available for many years. It only remained for dairy technology to work out the details for a commercially feasible process.

Demmler,[93] using whey and a mixed culture containing predominantly *Torula utilis*, reported on a continuous process for the production of yeast in high yields. The fermentation was conducted in a Waldhof-type fermentation tank equipped with a rotating sparger. Under normal operating conditions, an average yield ranging from 13 to 15 gm. of yeast per liter whey was obtained. In addition, 1.24 gm. of heat-coagulable whey proteins were obtained in association with the yeast. The drum-dried yeast product contained 59.4% protein, 4.7% fat, 26.6% invert sugar, 9.2% ash, 3.17–3.4% P_2O_5,

.6% moisture, and 0.2% sulfur. The purine content was lower han the average content in other yeasts. The drum-dried product vas more digestible than the spray-dried, presumably because f destruction of the cell walls in the drum-drying operation. Iowever, a preliminary heat treatment prior to spray processing re- ıoved this difference.

The lactose-fermenting yeasts contain the following vitamins in ıilligrams per cent on a dry basis: vitamin A, traces; B_1, 12.8; B_2, .4; nicotinic acid, 8.3; ascorbic acid, 7.8; and provitamin A, 0.5.[468A]

The mixed culture used by Demmler was derived from a process tilizing sulfite pulp waste liquor. The predominant organism, *T. tilis*, was not active toward lactose. Other organisms present in the ulture presumably were responsible for the fermentation in whey. 'hese required aeration for both growth and alcohol production. 'hey required iron, boron, manganese, potassium, magnesium, sul- ır, and phosphorus—all of which are present in whey.

Porges *et al.*,[406] studying the problem of waste disposal, obtained ıteresting data in connection with yeast production. *S. fragilis* was elected as the lactose-fermenting yeast most suitable for further tudy. In a laboratory batch process, with a 0.1% dispersion of kimmilk solids, *S. fragilis* assimilated 78% of the lactose and 78% of itrogenous compounds. The addition of 113 ppm nitrogen in the ɔrm of ammonium sulfate per ten parts nonfat milk solids per liter ıcreased lactose utilization in a 24-hr. period from 30 to 98%, and east production in the range of 17- to 42-fold. Proportionate quan- ties of ammonium sulfate added to solutions containing 25 and 50 m. nonfat milk solids per liter brought about respective increases in east production ranging between 13- to 22-fold and 12- to 20-fold. Vith a 12-liter Humfeld fermenter, employing a 1-liter inoculum, nd 2.5% nonfat milk solids or 2.5% whey solids, each supplemented ith 281 ppm nitrogen, Porges *et al.* obtained yields based on solids resent at the start in a 13- to 16-hr. period of 40% yeast (including ısein solids) from nonfat milk solids, and 20% yeast from whey ɔlids. In a continuous fermentation, employing a 2-liter fermenter,) gm. whey solids, 1,060 ppm nitrogen in the form of ammonium ılfate, and 750 ml. starter culture grown *in situ*, they recovered east in 24% yield based on original solids present from 2 liter whey. ased on available sugar, yields of yeast of 35 and 29% were ob- ıined with *S. fragilis* and *T. cremoris* respectively, in an experiment ith clarified whey.

The yeast fermentation has recently been brought to a high degree

of perfection and placed on a reasonable economic basis.[522–525] Peak oxygen requirements of 100–120 ml. O_2 per liter whey per min. corresponding to a solution rate of 1 lb. per min. were realized in both laboratory and plant investigations in which specially designed sprayer-agitation combinations were employed.[520–525] Supplementation of whey in laboratory experiments with 0.5–1% ammonium sulfate, 0.5% dipotassium phosphate, and 0.1% yeast extract together with the employment of a heavy inoculum constituting 25–30% of the weight of sugar present resulted in both maximal assimilation of available carbon and nitrogen, and maximal assimilation rates. Thus the rate was reduced from the usual 12–24 hr. to 3–4 hr. without impairment of yeast yield or quality (high protein content). Calculation based on the quantity of lactose and lactic acid carbon convertible to yeast carbon showed that a theoretical yield of 27 gm. of yeast (containing 45% carbon) per liter of whey was possible. Actual yields 85% of theoretical were obtained. Stated otherwise, about 0.55 lb. dry yeast could be obtained per pound of lactose.

Based on laboratory findings, subsequent escalation of operations to a pilot plant scale employing an 800-gal. propagator was carried out successfully.[522,523] The following table shows a representative balance sheet for the growth of *S. fragilis* in a whey medium.

TABLE 101

REPRESENTATIVE BALANCE SHEET FOR THE GROWTH OF *S. fragilis* IN WHEY MEDIUM IN PILOT PLANT EQUIPMENT[a]

	Batch 1	Batch 2
Volume, whey, gal.	450	600
Lactose (4.86% of whey), lb.	182	242
Lactose disappearing, lb.	182	242
Volume seed yeast, gal.	81	150
Final volume in tank, gal.	530	800[b]
Gross weight of yeast yield (dry), lb.	140	215
Net weight of yeast yield, lb.	74	105
Theoretical yeast yield (55% of sugar weight), lb.	100	133
% theoretical	74	79

[a] From data of Wasserman, *et al.* [523]

[b] 50 gallons water added accidentally.

Exceedingly important in the yeast fermentation are the propagators with their aerator-agitator combinations. Upon these and their operation depends the oxygen absorption rate of the medium which must correspond at least to the peak oxygen demand of the growing culture. The problem of design has been considered by Wasserman and Hampson who observed a dependency of the oxygen absorption rate on agitator design and speed and aeration rate.[521] With the Waldhof fermentor, good growth was obtained even when

he desired oxygen absorption rate 5 millimoles O_2 per liter per min. vas not realized.

Of the nitrogenous constituents in whey, it appears that yeast tilizes the ammonia nitrogen and about two-thirds of the heat non-oagulable organic nitrogenous compounds to the exclusion of the eat coagulable nitrogenous substances.[519]

In reproducing its own substance, the yeast cell produces an bundance of nucleic acids. Thus, not all of the nitrogen in yeast is rotein nitrogen although calculation of protein concentration is ased on this assumption. An estimated 20–40% of bacterial nitrogen considered to belong to nucleoproteins.[24]

Amino Acid and Vitamin Composition of S. fragilis.—*S. agilis* can be grown in whey to contain 50% protein (assuming all f the nitrogen is protein nitrogen) with an amino acid composition iffering very little from yeasts in general. Of the total amino ids in dried cells an appreciable fraction (28% nitrogen) is ex-actable when the cells are treated successively with trichloracetic id, ethyl alcohol, ether-alcohol, and trichloracetic acid. Histi-ne is largely extractable whereas serine and valine remain with the otein fraction. The quantity of amino acids in the extractable ıt not the protein fraction will increase if the yeast is grown in edia supplemented with 1% $(NH_4)_2SO_4$, and in general the quan-y and composition of extractables are influenced by yeast strain, e composition of the medium and the age of the yeast. The amino ids in the protein fraction are rich in lysine, aspartic acid, and utamic acid and are deficient in sulfur containing compounds. ginine, threonine, serine, glycine, alanine, valine, isoleucine, and rosine are found in respectable and nominal concentrations.

Wasserman found the following vitamins and their concentrations micrograms per gram in *S. fragilis:* thiamine 24.1, pyridoxine 13.6, oflavin 36.0, niacin 280.0, folic acid 5.8, pantothenic acid 67.2, *p*-ıinobenzoic acid 24.2, biotin 2.0, choline 6,710, and inositol 3,000.

ıctose in the Production of Penicillin

The reason for the startling increase in demand for lactose during orld War II was, of course, the discovery that this carbohydrate s uniquely suitable for the production of penicillin in high yields. e demand for lactose continued to increase largely in connection h an expanding penicillin industry. The competitive position lactose has been weakened in recent years by the discovery that trin can be used as a substitute, and that with adequate control pH other substrates may be used to advantage.

High yields of penicillin are obtained with the molds, *Penicillium notatum* and *Penicillium chrysogenum*, under conditions in which rapid mycelial growth occurring during the initial phase of the fermentation is followed by a period of slow fermentation. The initial phase can be accelerated by the addition of 0.5% glucose to the usual medium containing 2% corn-steep liquor and 2% lactose.[252] Jarvis and Johnson[226] found that optimum yields were obtained with a medium containing three parts lactose per one part glucose. The advantage accruing to the use of lactose is considered to reside in its slow utilization by the fungus. Since the lactic acid in corn-steep liquor is oxidized prior to the oxidation of lactose, the increase in pH is more rapid; and autolysis of the mycelium is retarded because of the longer time required for the exhaustion of nutrients. The inferiority of glucose is related to unfavorable factors resulting from its too-rapid utilization. These are: (1) slow rise of pH during penicillin production; and (2) premature exhaustion of carbohydrate and of ammonia nitrogen, leading to untimely autolysis and a cessation of penicillin synthesis.

Moyer and Coghill[359] were the first to state that yields of penicillin in corn-steep liquor media were relatively small when media containing glucose were compared with those containing lactose. This observation was later[472,475] confirmed.

With lactose-glucose media, Jarvis and Johnson[226] found that utilization of acetate proceeded at the same rate as utilization of ammonia, and as a consequence the pH remained constant during the period of their utilization. With the disappearance of glucose, utilization of acetate proceeded at a relatively greater rate, and the pH rose. Lactate was metabolized much less rapidly than ammonia during the decomposition of glucose, and only slightly more rapidly during the decomposition of lactose. Applying these observations, Jarvis and Johnson found that it was possible to achieve adequate control of pH during fermentation by the simultaneous use of acetate and lactate in lactose-glucose media.

Whey has been utilized with good results in the production of penicillin by surface culture methods.[11] Its commercial utilization as a source of lactose in submerged cultures is objectionable on several grounds—foaming becomes difficult to control, and the enrichment and purification procedures become unduly involved.

Microbiological Synthesis of Nisin

Nisin is distinguished from the vast majority of antibiotics in that it is an assimilable polypeptide which can be tolerated in large dosage

by humans, and appears to be without influence on the intestinal flora. It is found in cultured milks in which pure *S. lactis* cultures are used as well as in raw milk and in milk products such as cheese. Great interest has centered around its faculty, when present in cheese, of minimizing (although not in all instances) blowing and inhibiting the butyric organisms from growing.[20,52,103,132,206,207,256,418,440,491]

Nisin has recently been applied successfully in preparing sterile beverage quality chocolate milk. Serving as a sterilization aid, nisin because it inhibits the outgrowth of heat-damaged spores permits the use of less drastic heat treatment in the sterilization step.[197]

Skimmilk is a suitable medium for the production of nisin.[190,540] It is inoculated with an active strain of *S. lactis*, and after 40 to 48 hr. during which pH values between 4.5 and 5.5 are established, the coagulated proteins containing nisin are separated by centrifugation. This preparation is useful commercially. It may be dried and the nisin extracted with acidified acetone. Methods for further purification are given by Falconer and Cheeseman and Berridge.[60] In a patent, Hawley and Hall[190] describe a process in which sterilized skimmilk is cultured with *S. lactis* until the titer at pH 6.0–6.3 reaches 1,000 Reading units per ml. The paracasein is precipitated with $CaCl_2$ and rennin, and the resulting whey is adjusted to pH 4.0–4.5 with HCl and drained. The combined whey and curd washings adjusted to pH 5.0 are transferred to a circulating system of vertical foam tubes and 0.1% Tween is added. The collected foam contains 40,000 Reading units per ml. Solid nisin is prepared by saturating 500 ml. foam with 27 ml. acetone. The resulting precipitate is extracted with 500 ml. methanol, and the nisin in the extract is precipitated with 1,000 ml. acetone. The dried precipitate has an activity of 1.4×10^6 Reading units per gm.

Production of Vitamins

Microbiological Synthesis of Riboflavin.—Three types of microorganisms are characterized by their ability to synthesize riboflavin in commercially significant quantities. Bacilli of the species *C. acetobutylicum* synthesize it in quantities up to 50 mg. per liter. Yeasts of the species *Candida guilliermondi*, and related species, synthesize it under suitable conditions in quantities exceeding 100 mg. per liter. Yeast-like fungi of the order *Endomycetales*, species *Ashbya gossypii* and *E. ashbyii*, are the most productive, and under proper conditions will synthesize it in quan-

tities as high as 2.4 gm. per liter. The *Eremothecium* and *Ashbya* fermentations have another decided advantage in that flavinogenesis is not inhibited by trace quantities of iron—a restriction encountered to an intolerable degree with *C. guilliermondi* and to a lesser degree with *C. acetobutylicum*. Riboflavin synthesis in the acetone-butanol fermentation by means of *C. acetobutylicum* has been discussed above in connection with the production of butanol.

C. guilliermondi and related species will not produce significant quantities of riboflavin in the presence of iron in concentrations greater than 0.1 ppm.[47] This limitation is a severe one, and practically limits the application of the *C. guilliermondi* fermentation to synthetic media.

Flavingenesis with E. ashbyii.—Of the related species, *E. ashbyii* and *A. gossypii*, only the former utilizes the nitrogenous substances of whey to effect the synthesis of riboflavin.[295] With *A. gossypii* and a suitable carbohydrate, luxuriant growth but practically no riboflavin synthesis is observed in whey media.[295]

Whey is not a complete medium for the growth of *E. ashbyii*. Neither lactose nor galactose is broken down by it, nor can it be adapted to lactose.[295] However, whey in solutions 0.1 *N* with respect to HCl, heated for 1 hr. at 120°C., followed by neutralization, yields a product which is quite suitable for supplementation of malt extract media, whey, mixtures of these, and presumably media containing one or more members of a large variety of nitrogenous substances. The fullest utilization of the nitrogenous substances of whey requires abundant and efficient aeration, and such aeration entails in practice a serious foam problem, which persists even with the addition of foam depressants.

Under conditions of mild agitation and aeration, the nonheat-coagulable nitrogenous constituents of whey constitute the most readily utilized nitrogen fraction. Efficient utilization of both carbohydrates and nitrogenous substances is optimal in media that contain relatively small quantities of these substances, that is, under starvation conditions. Of course this entails poor yields on a volume basis, yet the results indicate that an advantage would accrue to the use of a continuous fermentation employing small, stationary concentrations of carbohydrate and nitrogenous substances.[295]

Maximum yields of 200 mg. riboflavin per liter whey were obtained by Leviton and Whittier[302] with hydrolyzed, clarified whey diluted with an equal volume of water. Much greater yields were obtained if hydrolyzed, clarified whey was used to supplement malt extract

as a source of carbohydrate and assimilable nitrogenous substances.[295]

Startlingly high yields have been reported. Examination of the experimental and cultural conditions under which these high yields were obtained discloses the use of media rich in dissimilable carbohydrates and nitrogenous substances, together with lengthy and intensive aeration. Phelps[395] reported yields of 650 mg. riboflavin per liter in media containing 8 ml. skimmilk, 1.75% malt extract, and 0.5% cerelose in 25 ml. Sjöström and Håkansson,[459] using the same culture employed by Leviton but modifying it by serial transfers, obtained yields as high as 400 mg. per liter, in clarified whey media supplemented with 1% sucrose.

Heyndrickx and deVleeschauwer[201] employed higher concentrations of ingredients and quite violent agitation. Average yields with whey containing 1, 2.5, and 5% sucrose were respectively 435, 665, and 755 mg. per liter. However, by using a mixture of equal parts of whey and skimmilk, and 1, 2.5, and 5.0% sucrose, they obtained average yields of 630, 1,150, and 1,575 mg. per liter, respectively. The maximum yield obtained with 5% sucrose in a whey plus skimmilk medium was 2,375 mg. of riboflavin per liter. A large number of patents have been issued defining various media which conduce to high yields.[225,358,441,474,479]

Cultural conditions which must be met in order to obtain reproducible results and high yields are: control of initial pH within the range of 5.4–7.0; preparation and transfer of inoculum according to a consistent and uniform plan; employment of temperatures within the range of 25–35°C.; and avoidance of overheating of heat-sensitive components during sterilization. Heyndrickx claims that no loss in activity results when inocula are stored 4–5 months at low temperatures, and that cultures 12–24 hr. old possess maximal activity.[200] Potassium benzyl penicillin added to the extent of 25 units or more per milliliter of medium prevented infection of pasteurized media during fermentation without impairment of yields.[199]

Riboflavin proportions itself between the mycelia and surrounding medium during the course of its synthesis.[295] The partition coefficient remains constant up to the point at which the mycelia disappears, and large numbers of spores appear extracellularly. At this point, practically all of the riboflavin together with large quantities of soluble nitrogenous compounds are excreted into the medium.

Cultures when plated on a yeast-extract-peptone-agar medium

yield pigmented and white colonies.[200,295,429] Serial plate transfers of cells from a pigment-free colony serve to perpetuate the substrain. Only occasionally is a pale yellow colony observed. Serial transfers of cells from pigmented colonies serve to perpetuate a mixture with the formation of increasing proportions of pigmented colonies, until a steady state is reached. However, temporary reversions have been observed from time to time in favor of the pigment-free strain. It is advisable, therefore, to inaugurate a fresh series of transfers from time to time either from pigmented colonies or from a lyophilized, productive culture. Temporary reversions have also been observed in the other direction in favor of the pigmented strain, and on occasions extraordinarily high yields are obtained which are difficult to reproduce.

Much work has been done with *A. gossypii*,[408,409] and detailed laboratory, pilot-plant and plant-scale procedures for the microbiological production of riboflavin have been published.[408]

Microbiological Synthesis of Vitamin B_{12}.—Microbiological synthesis affords the only means known at present for the bulk production of pure vitamin B_{12} and vitamin concentrates containing it. A number of reports concerned chiefly with vitamin B_{12} yields in actinomycetes cultures appeared between 1949 and 1951.[133,134,161,162,451]

A strain of *Bacillus megaterium* was found active with suitable substrates, among which whey was one. Garibaldi *et al.*[134] obtained yields of 0.8 mg. per liter, corresponding to a glucose consumption of 10 gm.

A low cobalt-ion concentration was shown by Hendlin and Ruger[198] to limit the synthesis of vitamin B_{12}. Cobalt comprises about 4% of the molecule. Working with 13 cultures, including a strain of *Streptomyces griseus*, unidentified rumen and soil isolates, a strain of *Mycobacterium smegmatis*, and *Pseudomonas* species, Hendlin and Ruger reported that the addition of 1–2 ppm of cobalt ions gave rise to approximately a three fold increase in yield.

Leviton and Hargrove[179,180,297,298,301] found that bacteria of the genus *Propionibacterium* elaborated vitamin B_{12}-active substances in concentrations equal to or greater than those reportedly obtained with other organisms. The active compound produced was identified as hydroxocobalamine.

They[300,301] compared lactose and glucose as sources of energy in a number of vitamin B_{12} fermentations. Employing several strains of *B. megaterium* and several unidentified rumen isolates, they found that lactose brought about higher yields and faster fermentation. Using *Streptomyces olivaceus* as the organism, and

clarified whey as lactose source, Leviton[295] compared lactose and glucose in enzymatically-hydrolyzed casein-yeast extract media, in distillers' soluble media, and in ammonium caseinate media. All media were fortified with Co^{++}. Higher yields were obtained with the lactose-containing media.

In laboratory scale experiments in which *L. casei* was used symbiotically with *Propionibacterium freudenreichii* in the fermentation of whey, the average yield was 2.2 mg. per liter and the maximum was 4.3 mg. per liter.[300]

The production of vitamin B_{12} compounds is not species specific. All species of the *Propionibacterium* genus, when cultivated under the same conditions, will produce active substances, yet in different quantities. *P. freudenreichii* and *Propionibacterium zeae* synthesized sufficient quantities to warrant their consideration for commercial exploitation. As propionic acid bacteria are active during Swiss-cheese ripening, it was anticipated, and actually has been demonstrated, that the production of vitamin B_{12} in Swiss cheese is influenced by the same factors which influence its production in pure culture, particularly by the cobalt content of milk.[179,180]

Propionic acid bacteria require, for maximal growth rates, a highly degraded source of amino acids. In caseinate media, and even in peptone media, the rates are apt to be relatively slow. For maximal yields of vitamin B_{12}, a high degree of anaerobiosis is not required. Because assimilation is largely anaerobic, a high ratio between vitamin concentration and total cell mass is obtained. Thus this fermentation is particularly suitable for the preparation of the pure vitamin, inasmuch as the cell mass contains the entire vitamin content and furnishes a highly concentrated initial source for further treatment. As a first step in further treatment, harvested cells may be coagulated and then lysed in a 50% by volume acetone solution, or in mixtures of butyl and ethyl alcohols.[298]

Sewage wastes have been shown to contain as much as four ppm of vitamin B_{12}.[102,214,350,351] Although frowned on for aesthetic reasons as a source of vitamin B_{12} for human nutrition, the wastes from activated sludge processes may well provide the cheapest source for the preparation of vitamin B_{12} concentrates for stock feed. Symbiotic growth of lactic and acetic acid bacteria has been recommended as a means for producing sour milk products, biologically enriched with vitamin B_{12}.[445] Acetic acid bacteria cultured in whey fortified with cobalt salts led to an 80-fold increase in vitamin B_{12}. Propionic acid bacteria in skimmilk supplemented with dimethylbenzimidazole[86] conduced to a 300-fold increase in the vitamin.

In view of the recent work of Barker *et al.* and Weissbach *et al.*, it appears likely that the natural cobamide produced in bacterial cultures is not vitamin B_{12} but rather coenzyme B_{12}.[17,18,526] The main difference in composition between the coenzymes derived from *Clostridium tetanomorphum* and *Propionibacterium* cultures and the corresponding vitamins and pseudovitamins is the absence of the cyano, or hydroxo groups and the presence instead of an adenine nucleoside in association with cobalt. The nucleoside contains adenine and a sugar-like compound characterized as D-erythro-2,3-hydroxy-Δ-4-pentenal linked to the N-9 position of adenine.[210] In assays with *E. coli* and *Ochromonas malhamensis*, the response to the vitamins and corresponding coenzymes is identical. The hydroxocobalamine vitamin B_{12} can be formed from the 5,6-dimethylbenzimidazolyl coenzyme B_{12}. Consideration of the yields reported by Barker *et al.*[18] for the enzyme and by Leviton[300] *et al.* for the vitamin suggests that the vitamin is derived from the coenzyme during the preparation of bacterial extracts for analysis.

The "Oxidative" Fermentations

Whey does not lend itself directly to the production of acetic acid by means of species of the genus *Acetobacter*. Furthermore, the use of combined inocula of yeasts and *Acetobacter* species has not proved fruitful. However, Haeseler[159] has described an operable procedure, in which an alcohol followed by an acetic acid fermentation yielded a vinegar with satisfactory qualities.

The production from whey of a vinegar with as much as 10% acid seems unlikely because of adverse effects from a high salt concentration. The production of a 5–7% acid vinegar from whey may prove feasible. However, in the process described by Haeseler, a whey vinegar containing only 4% acid was produced. This product, yellow-brown in color, had a malt-vinegar character with only a weak whey taste and a slight saltiness, which were not detrimental. The possibility of slime formation and over-oxidation with whey as a substrate was considered detrimental to the use of quick vinegar processes.

A process for making a vinegar substitute from whey has been claimed in a French patent.[494]

The production of lactobionic acid from lactose, through bacterial oxidation of the aldehyde group, is of some interest in connection with the interesting properties which the product possesses. Lockwood and Stodola,[315] using *P. graveolens*, recovered lactobionic acid in 77% yield from a fermentation mixture containing the following

components per liter: 96 gm. anhydrous lactose, 0.62 gm. KH_2PO_4, 0.25 gm. $MgSO_4 \cdot 7H_2O$, 2.1 gm. urea, 28 gm. $CaCO_3$, 5 ml. corn-steep liquor, and 0.3 ml. soybean oil.

Kluyver *et al.*[248] have described a *Pseudomonas* species which produces large yields of lactobionic acid. Villecourt and Blachére[501] have reported on a bacterium, *Bacterium anitratum*, which, although it does not utilize lactose, oxidizes it.

The sequestrant and emulsifying properties of lactobionic acid suggest a commercial potential for this product. In addition, it is a solubilizing agent for calcium salts. Solutions of calcium lactobionate containing as much as 70% salt have been prepared. This product may prove valuable in the pharmaceutical trade as a source of calcium.[236]

Other Fermentations Using Whey

The making of whey-cheese is, perhaps, one of the earliest of fermentations which used whey (or its components) as a substrate. Examples of such cheese include Schottengsied, Primost (Mysost), Ricotta, and Gjetost (made from goat's milk whey).

Whey has been suggested as a culture medium for growth of lactic bacteria. Czulak[83A] reported whey could be used to grow *P. roqueforti*. Recently Lundstedt and Fogg[319A] found whey suitable for growth of *S. diacetilactis*. They noted further that when citrated whey was cultured with *S. diacetilactis* and added to creamed Cottage cheese, a pleasing diacetyl flavor and aroma developed in 2 to 6 days while the cheese was held under refrigeration.

Use of fermented whey as a food has been suggested. Jagielski[224A] combined whey and lactose with an appropriate culture and produced a whey kumys. More recently Krul'kevich[241A] mixed equal volumes of whey and buttermilk with kumys yeasts, *L. bulgaricus* and *L. acidophilus*. The finished product is claimed to resemble kumys. A condensed whey food comprised, in part, of whey fermented by *L. bulgaricus* and *P. shermanii* has been described in a patent issued to Meade *et a.*[339A]

Other uses for whey based on fermentation include production of: (a) lactase enzyme from *S. fragilis* (or other organisms able to utilize whey) as described by Myers and Stimpson;[361A] (b) a high vitamin, high protein product containing little or no lactose and prepared by fermenting whey with an organism able to utilize lactose (e.g., *S. fragilis*) followed by drying the fermented material;[361B] and (c) an animal feed suitable for ruminants by fermenting whey with *L. bulgaricus* at a pH of 5.8–6.0, concentrating the fermented whey to

30–80% solids and neutralizing the concentrate to pH 7–8.[60]

Attempts to improve the quality of whey include those of Johnstone and Pfeffer,[230A] who increased its nitrogen content with a nitrogen-fixing strain of *A. aerogenes*, and Davidov and Rykshina,[86] who used whey fortified with $CoCl_2$ and, after fermenting it with acetic acid bacteria, observed an 80-fold increase in vitamin B_{12}.

The addition of a whey paste plus a nisin producing strain of *S. lactis* to silage has been suggested by Zeilinger and Binder[551A] as a means to prevent development of butyric acid bacteria in the fodder.

DAIRY WASTE DISPOSAL

The wet oxidation of dairy waste is one of the most serious and strenuous tasks microorganisms are called upon to perform. The microbiological system must oxidize the carbon and hydrogen of organic compounds to carbon dioxide and water, respectively, and must at the same time conserve its own mass. In other words, the cellular mass must neither increase nor decrease over long periods of time. That this ultimate objective is closely approached in practice testifies to the remarkable power of the metabolic apparatus of microorganisms.

Dairy wastes fall into two categories one of which may be described as an intrinsic, and the other as a conditional waste. All dairy plants experience losses which are intrinsically a part of plant operation. For example a small dairy plant that receives 10,000 lb. of milk daily, may produce each working day about 1,250 gal. of waste with a milk solids concentration of 0.1%. Cheese plants on the other hand, produce as a by-product of cheese-making, whey, which although it contains half of the nutrients of the milk from which it was derived must be treated as a conditional waste—conditional upon the absence of a suitable market for its use. It has been estimated that some 80% of cheese plants have whey disposal problems. The principles elaborated for the aeration of wastes are quite general and apply to all kinds of dairy wastes.

Dairy Waste Treatment by Aeration

The magnitude of the chemical, or biological oxygen demand of solutions of organic matter determines whether or not these solutions may be safely added to sources of marine life. Chemical oxygen demand (COD) is the amount of oxygen, determined chemically, necessary for the complete oxidation of an organic substance, and is usually reported in parts per million (ppm).[405] It is practically equal

for milk wastes to the ultimate biochemical oxidation demand (BOD).

As oxidants either permanganate or dichromate may be employed under standard conditions of concentration, temperature, and time. These reagents recently have been studied critically; only the results with dichromate were found to reflect accurately the BOD of dairy wastes.[127]

Aeration techniques will be successful only if oxygen can be supplied at a sufficiently high rate to lower the COD to acceptable values.[265] Extensive investigations on the bio-, and chemical oxidation of dairy wastes have shown that each pound of dry organic matter in dairy waste requires about 1.2 lb. of oxygen for complete oxidation.[212,213,402] During the period of rapid assimilation bacteria need about 37.5% of their complete oxygen requirement, or 0.45 lb; and in the process, 0.52 lb. of new cell material is formed per lb. of waste solids. To oxidize this newly formed sludge, 0.75 lb. oxygen is required, the difference between the oxygen required for complete oxidation of one lb. of waste solids and that required for assimilation. During endogenous respiration at 32.2° C, sludge is consumed at an hourly rate of approximately one per cent.[217] Thus, if an amount of sludge equal to 0.52 lb. of newly formed cells is to be oxidized in the time t_1 no less an amount of sludge than that given below would be required to maintain this condition:

Equilibrium weight of sludge per lb. organic matter $= 52/t_1$. If the parts oxygen required to oxidize the organic matter in one million parts of waste volume—the ppm COD—is known, the total oxygen requirement in pounds for any given waste volume, V, in gallons, is easily calculated. The weight of organic solids is equal to 83.3% of the total oxygen requirement (COD) and hence the equilibrium sludge weight is given by the following equation, thus: sludge $= (52 \times V \times \text{ppm COD} \times 8.34 \times 0.833 \times 10^{-6})/t_1$.

If for example, a waste volume, V, of 10,000 gallons with a ppm COD of 1,500 is to be processed in $t = 20$ hr., the equilibrium sludge weight would be 270 lb. The calculation is oversimplified and is about 10% too low assuming as it does, that endogenous respiration and assimilation occur simultaneously during the entire operation. Actually, there is always a retention time during which cellular substance is consumed without replenishment.

The hourly oxygen requirement for sludge respiration is equal to the sludge dissipation rate multiplied by the lbs. of oxygen (1.44) required for the oxidation of each lb. of ash-free sludge. The hourly oxygen requirements for assimilation is given by the quotient of

total oxygen required for assimilation and the time required to introduce the waste. The hourly oxygen requirement during assimilation is equal to the sum of the two aforementioned requirements, and may be expressed in terms of the volume V, of influent, the ppm COD, the feed time, t_2, and the endogenous respiration time t_1, thus: O_2 (lb. per hr.) = $(5.2\ V \times \text{ppm COD} \times 10^{-6})/t_1 + (3.13\ V \times \text{ppm COD} \times 10^{-6})/t_2$.

The equation summarizes some of the arguments and the data contained in the literature.[404] The aeration device must be designed to furnish the solution with oxygen at the required rate. The tank must be designed to accommodate the milk waste and the sludge. Allowances must be made for a certain proportion of free space (freeboard), and settling space. The design, construction and operation of dairy waste disposal units have been considered theoretically and practically by workers in the United States Department of Agriculture.[401,404]

Processing of Whey Wastes

Whey solids compared with milk solids contain a greater proportion of lactose, and a much smaller proportion of nitrogen. Consequently in the processing of whey wastes even under conditions of adequate aeration, the rate of assimilation may be limited by the COD-nitrogen imbalance. Jasewicz and Porges[227] observed that when sludge (2,000 ppm COD) was used to treat dilute whey waste (1,000 ppm COD) under highly aerobic conditions no additional nitrogen was necessary for complete whey removal, inasmuch as the essential nitrogen was supplied in the course of endogenous respiration. Addition of ammonium sulfate to aerators was recommended to compensate for the additional load imposed on them when whey is wasted along with the normal load. In pure whey studies it was found that under the laboratory schedule of daily feedings both supplemented and unsupplemented sludges gradually deteriorated, and presented serious bulking problems after three months. This was taken to indicate that supplementation with nitrogen alone was not enough. Pursuing the problem further, Porges and Jasewicz[403] in a 61 day study of the COD balance in a system to which whey was added 48 times to aerated sludge observed that whey wastes may be readily treated under certain conditions without nitrogen addition. An average of 75% of the influent whey COD was relieved, when no provisions were made for the removal of sludge from the effluent. The sludge accounted for all but 2–3% of the effluent COD. Calculation

based on a sludge oxidation rate of 6.3% per day showed that dynamic equilibrium would be possible if 100 units of sludge were used to treat 10 units of whey.

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